University of Bath

PHD

Immunological studies in motor neurone disease

Aspin, Jacqueline Patricia

Award date:
1986

Awarding institution:
University of Bath

Link to publication

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal ?

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 13. May. 2019

IMMUNOLOGICAL STUDIES IN MOTOR NEURONE DISEASE

Submitted by Jacqueline Patricia Aspin

for the degree of Ph.D.

of the University of Bath

1986

COPYRIGHT

Attention is drawn to the fact that copyright of this thesis rests with the author. This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with the author and that no quotation from the thesis and no information from it may be published without the prior written consent of the author.

This thesis may be made available for consultation within the University Library and may be photocopied or lent to other libraries for the purposes of consultation.

UMI Number: U001269

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

UMI U001269
Published by ProQuest LLC 2014. Copyright in the Dissertation held by the Author.
Microform Edition © ProQuest LLC.
All rights reserved. This work is protected against
unauthorized copying under Title 17, United States Code.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

UNIVERSITY OF BATH
LIBRARY
26 | 15 SEP 1987 |
PHD

Acknowledgements

I wish to express my appreciation for the help given to me by my supervisors Dr. R. Harrison and Dr. G. Lunt. My deep gratitude for all his help and kindness to Dr. A. M. T. Jehanli and many thanks to all members of the Department of Biochemistry for their cooperation.

I also wish to thank my husband for his continued support and encouragement throughout the past three years.

I am indebted to the S.E.R.C. for a research studentship.

" Tell people what it
is like this Motor
Neurone Disease. Then
maybe someone will find
a cure. Then it won't
all have been in bloody
vain."

~ David Niven.

To my mother.

Abbreviations

CON A : Concanavalin A

PHA : Phytohaemagglutinin

PWM : Pokeweed Mitogen

PBS : Phosphate Buffered Saline

TBS : Tris Buffered Saline

FCS : Fetal Calf Serum

DHS : Donor Horse Serum

LCM : Lymphocyte Culture Medium

SFM : Serum Free Medium

SSM : Serum Supplemented Medium

MEM : Minimal Essential Medium

DMEM : Dulbecco's Modified Essential Medium

HAT : Hypoxanthine, Aminopterin, Thymidine

HT : Hypoxanthine, Thymidine

DNA : Deoxyribonucleic Acid

RNA : Ribonucleic Acid

AP : Alkaline Phosphatase

HRPO : Horse Radish Peroxidase

ELISA : Enzyme Linked Immunoabsorbent Assay

CELIA : Competitive Enzyme Linked Immunoabsorbent Assay

MND : Motor Neurone Disease

MS : Multiple Sclerosis

EBV : Epstein-Barr Virus

S.D. : Standard Deviation

O.D. : Optical Density

CPM : Counts Per Minute

SUMMARY

1) Conditions for the stimulation of peripheral blood lymphocytes with Con A, PHA, and PWM have been developed for 5 assay systems. Sub-optimal mitogen concentrations were established for the incorporation of [^{3}H]-thymidine, [^{3}H]-leucine, and [^{3}H]-uridine, into DNA, protein and RNA respectively, and for assays based on the release of lactate and glucose uptake. Stimulation of lymphocytes from MND patients by either Con A or PHA was found to be significantly depressed relative to normal age-matched controls as shown by the incorporation of [^{3}H]-thymidine or [^{3}H]-leucine or by glucose uptake. Stimulation of lymphocytes from MS patients by either Con A and PHA was also found to be significantly depressed compared to age-matched healthy controls. The incorporation of [^{3}H]-thymidine, [^{3}H]-leucine, the uptake of glucose and the release of lactate were all found to be significantly depressed.

2) Neurone-rich cultures from rat spinal cord cells were established. The cells were cultured in serum-supplemented medium for the first 3 days and subsequently in serum-free medium. The neuronal cells matured up to day 15-20 with large cell clusters and thick processes. These clusters and processes were labelled clearly with tetanus toxin which is a neurone cell marker. Membranes were prepared from 15-day old rat spinal cord cell cultures and used to stimulate peripheral blood lymphocytes

from MND patients and normal age-matched controls. There were 4 out of 14 MND patients that showed a blastogenic response compared with 0 out of 9 normal controls.

3) Peripheral blood lymphocytes from healthy donors were stimulated with Con A, PHA or PWM in serum-free medium. Stimulation with Con A and PHA was not significant with all 5 assays (previously described). However, with PWM, stimulation indices were significant ($p < 0.01$).

Lymphocytes from MND patients and normal healthy controls were cultured with rat spinal cord. Lymphocytes from 3 out of 3 MND patients were significantly stimulated compared with those of 0 out of 3 controls.

4) An ELISA was established for detecting anti-neural membrane antibody-producing hybridomas from MND lymphocytes. It involved coating individual wells with rat spinal cord membrane fragments. A similar assay for monoclonal anti-myelin basic protein antibodies was developed for MS-derived hybridomas.

5) Lymphocytes from MND, MS and normal individuals were fused with the mouse myeloma cell line P3. X63-Ag8-653. Normal and MND lymphocytes yieded hybridomas in 50% of the wells whereas the corresponding figure for MS lymphocytes was only 11%. 9% of the MND colonies and 2% of the MS colonies were found to be positive for their respective antibodies. These remained stable after recloning for 3 months. The principal immunoglobulin secreted was

of the IgG class.

Stimulation of MND, MS and normal lymphocytes with PWM for 5 days prior to fusion. Stimulation increased the number of cells for fusion by approximately 20%. However, this did not alter the percentage of hybridomas formed, except in the MS samples where the percentage rose from 11% to 50%. Similar numbers of hybrids secreted IgG and were positive for their respective antibodies. The colonies were recloned by limiting dilution and remained stable for over 3 months.

Lymphocytes from MND, MS and normal donors were initially stimulated with PWM for 5 days and then transformed with Epstein-Barr virus. It was found that 90% of wells contained colonies, which grew quickly and equal numbers of colonies secreted IgG or IgM. The number of positive colonies was 9% in MND and MS patients. The clones remained positive for over 3 months.

The culture supernatants which were positive with the ELISA were tested for binding to cultured rat spinal cord cells by immunofluorescence. There was no detectable binding of immunoglobulin to the cultures by immunofluoresence even after concentrating the culture supernatants by salt fractionation.

CONTENTS

CHAPTER 1

SECTION 1 THE IMMUNE SYSTEM

Section 1 The Immune System

The essential function of the immune system is defence against infection and the complexity of the system derives from an intricate communications network capable of exerting multiple effects based on relatively few distinct cell types. A normally functioning immune system is an effective defence against foreign agents such as bacteria, and against native cells which transform into tumour cells. The system produces a specific reaction to each infectious agent which prevents reinfection later.

The immune system comprises a variety of molecules and cells which are distributed throughout the body.

1.1. Components of the Immune System

1.1.1. Cellular Components

The differentiated cells of the lymphoid system, macrophages and lymphocytes, have their origin in pluripotent stem cells present in bone marrow. Lymphoid cells that undergo differentiation and acquire immunocompetence in the thymus give rise to re-circulating populations of mature T-cells. T-cells take part in a wide range of cell-mediated immune reactions which include protection from infection by fungi and viruses, graft rejection, and immune surveilance (Gupta, 1980). They comprise 70% of peripheral lymphocytes (Gupta & Good, 1980) and can be recognised by their capacity to form rosettes with sheep red blood cells (Aiuti et al, 1975; Taylor et al, 1977) and by the

presence on their membranes of targets for specific monoclonal antibodies (Haynes, 1981). They are concerned with the regulation of other lymphoid cells in the immune system and can be divided into effector and regulatory cells.

Effector T-lymphocytes are responsible for such functions as cytotoxicity and lymphokine production. Cytotoxic T-cells are produced, for example, when the host cells are infected with virus, or when a foreign tissue graft is introduced. Surface receptors of cytotoxic cells recognise transplantation antigens on the target cell, bind to the latter, and within a matter of minutes, bring about cytolysis (Cosimi, 1981; Ellis et al, 1982). Lymphokines are biologically active soluble factors some of which influence the movement and activity of macrophages.

Regulatory T-cells are divided into two major subclasses. T-helper (Th) and T-suppressor (Ts) which are involved in the regulation of antibody production by B-cells. Ts cells represent about 35% of the peripheral T-cells in a normal individual and inhibit the differentiation of B-cells into antibody-secreting plasma cells. Antigen-specific suppressor T-cells regulate the clonal expansion of B-cells by sending an inhibitory signal (Roitt et al, 1985) as shown in Figure 1. All T-cells recognise antigen but secrete only mediator substances. Th cells represent about 65% of peripheral T-cells and can also induce T suppressor or cytotoxic cell function (Reinherz and Schlossman, 1980). This

is shown in Figure 2. T helper and T suppressor can be identified by monoclonal antibodies (Table 1.)

TABLE 1. HUMAN MONOCLONAL ANTIBODIES REACTIVE WITH LYMPHOCYTES

MONOCLONAL ANTIBODIES	% LYMPHOCYTES	CELL TYPE LABELLED
Anti-T101, Anti-T, Anti-Leu 1	55-80	Most T-cells
Anti-T3, Anti-Leu 4	55-80	Most T-cells
Anti-T11, Anti-Leu 5	60-85	All T-cells
Anti-T4, Anti-Leu 3	40-60	Helper
Anti-T8,Anti-Leu	20-40	Suppressor T

(Haynes, 1981)

Activated Macrophages	Cytotoxic T-cells	Antibody Forming Cells
Macrophages	Pre-killer Cells	B-cells
	Helper T-cells	
	Suppressor T-cells	

Figure 1. Negative Feedback of Suppressor T Cells

Maturation of lymphoid cells in the bone marrow produces B-cells, a fundamentally different class of immunocompetent lymphocytes which can be recognised by their membrane bound immunoglobulin (Ig) (Baran, 1985). When triggered by the appropriate antigen, the B-cells differentiate further into memory cells, antibody-producing plasma cells, and regulatory B lymphocytes, which also secrete antibody. This lymphocyte population represents 10-20 % of peripheral blood lymphocytes in a normal individual. Antibodies secreted by the B-cells serve as protection against bacterial, and some viral infections (Gupta & Good, 1980).

None of these functionally distinct populations act in isolation but must cooperate with macrophages, antibody and each other to regulate the immune response (Figure 2).

1.1.2. Humoral Components of the immune system

1.1.2.(a) Antibodies

Antibodies are the best characterised molecules of the immune system and are produced by B-cells following their activation

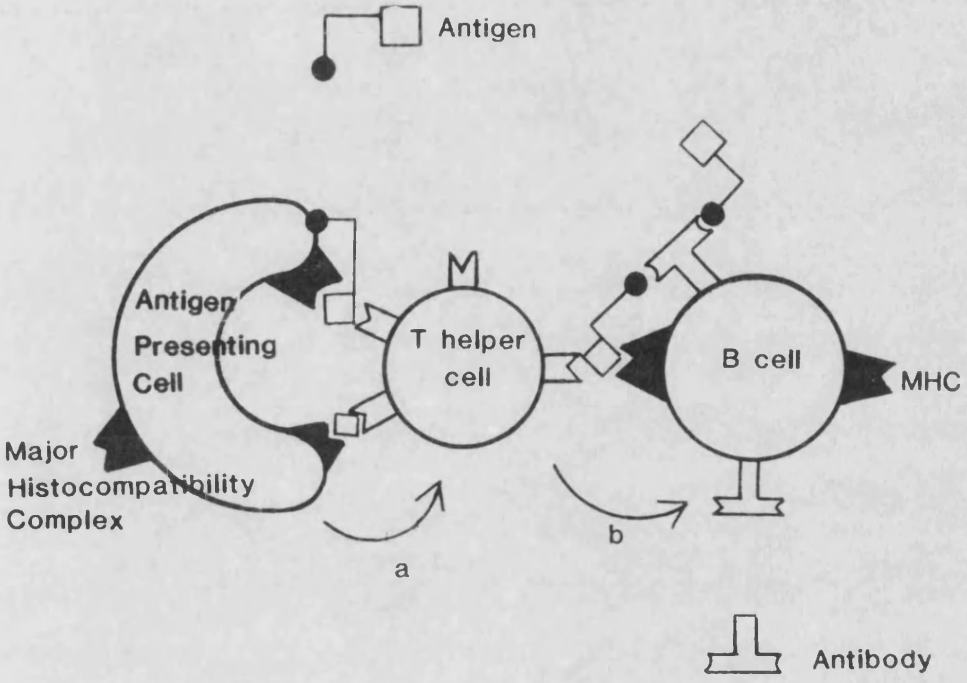


Figure 2 Antigen Presentation

A cell "presents" the antigen plus the major histocompatibility complex M.H.C. (a). This activates a helper T cell with a suitable receptor. The helper T cell sees the same antigen-MHC combination on a B cell (b). Here the antigen is held by surface antibody, and the interaction triggers the B cell to produce lots of antibody thus neutralizing circulating antigen.

into plasma cells. Five classes of heavy chains are found in human sera (IgG, IgM, IgA, IgD, IgE) and two light chain classes (kappa and lambda) Table 2 lists some of the characteristics of the immunoglobulins.

TABLE 2 CHARACTERISTICS OF IMMUNOGLOBULIN

	IgG1	IgG2	IgG3	IgG4	IgM	IgA1	IgA2	IgD	IgE
Heavy chain	G1	G2	G3	G4	M	A1	A2	D	E
Mean Serum Concentration mg/ml	9	3	1	0.5	1.5	3	0.5	0.03	5x 10-5
Sedimentation Constant	7S	7S	7S	7S	19S	7S	7S	7S	8S
Molecular Weight (x1000)	146	146	170	146	970	160	160	184	188
Number of heavy chain domains	4	4	4	4	5	4	4	4	5
Carbohydrate (%)		2-3			12	7-11		9	12

Antibody molecules have three main functions, complement-mediated cell lysis, antibody-mediated cell cytotoxicity, (via killer cells), and direct agglutination of particular antigens (e.g. bacteria) followed by removal of the complex by macrophages. Immune complexes are formed when antibody cross-links with antigen and they are involved in the regulation of the immune response.

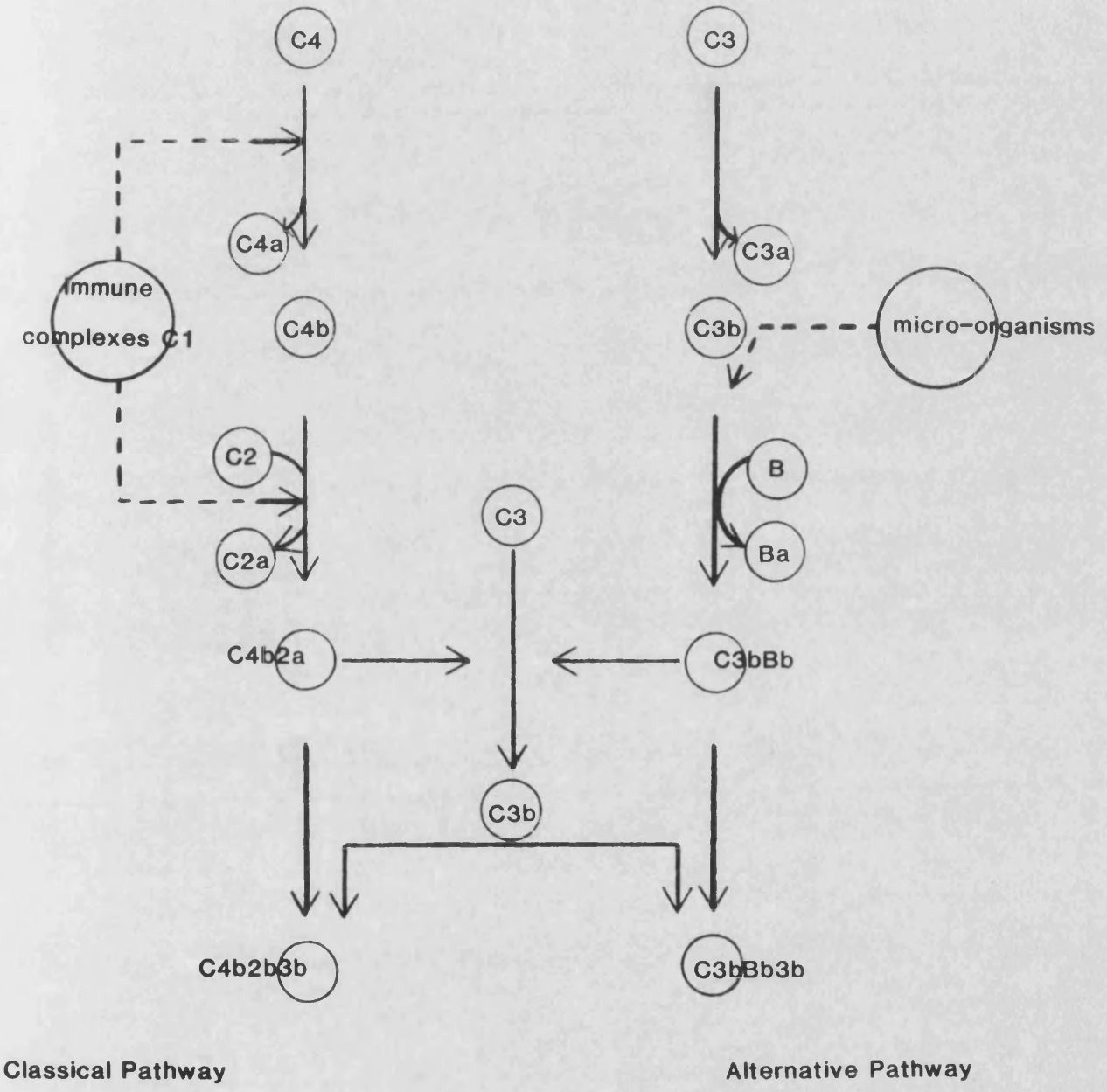


FIGURE 3 The Pathways of Complement

1.1.2.(b) Complement

Complement consists of a complex series of proteins many of which are proteinases. These proteins form two interrelated enzyme cascades termed the classical and alternative pathways (see Figure 3.), both providing different routes to the cleavage of C3. The enzyme cascades are generated by the activation of enzyme precursors which are, in turn, fixed to the membrane surface. Each enzyme precursor is activated by the previous component of complement which proteolytically converts the precursor to its catalytically active form. Each step is amplified as each enzyme activates many enzyme precursors. The complement system performs three main functions: cell activation: cytolysis: and opsonisation for phagocytosis.

1.1.2.(c) Lymphokines

Antigen-activated T-cells release mediators known as lymphokines. These are also released by B-cells, although more is known about those released from T-cells . There are many lymphokines with different functions. These are summarised in Table 3.

TABLE 3 FUNCTIONS OF LYMPHOKINES

FUNCTIONS OF LYMPHOKINES	:	LYMPHOKINE
1) Regulation of other lymphocytes	:	Interleukin-2 (IL-2)
	:	Interleukin-3 (IL-3)
	:	Interferons
	:	Soluble immune response suppressor
	:	T-cell replacing factor
2) Regulation of other lymphocytes	:	Assorted antigen specific helper factors
	:	Assorted antigen specific suppressor factors
3) Induction of Inflammation	:	Skin reactive factor
4) Modulation of phagocytes	:	Migration Inhibition factor
	:	Macrophage inhibition factor
	:	Chemotactic factor
	:	Macrophage fusion factor
	:	Colony stimulating factor
5) Regulation of other tissues	:	Colony stimulating factor
	:	Osteoclast activating factor
6) Destruction of non-leucocyte cells	:	Lymphotoxins

1.2. The Immune System and the Central Nervous System

1.2.1. CNS; an immunologically privileged site

For many years the brain was regarded as being inert immunologically. These ideas stemmed from experiments carried out early in this century with transplantations and viruses. Grafts in the brain were found to be more successful (Murphy and Slurm, 1923), even cross species, and viruses were able to proliferate unhindered in brain tissue (Theiler,1930).This led to the idea that the brain was not under immune surveillance with the rest of the body but was surrounded by a barrier preventing the entry of antibody and immunocompetent cells.

These views have now been modified. It is now known that the brain is only partially privileged and that most of the effector cells involved in the immune response are present in low numbers within the CNS (Traugott et al, 1981; Aarli, 1983). Pluripotent stem cells have even been reported (Bartlett, 1982; Phillips et al, 1982). However, the proportions of different immunocompetent cells in the brain are not the same as in the peripheral blood or lymphoid organs (Circolo et al, 1982). The brain can reject grafts (Scheinberg et al, 1964) and can elicit immune responses (De Micco et al, 1986). There is no organised lymphatic system in the brain indicating that the brain is not normally exposed to antigenic attack (Darling et al, 1981). The brain is a partially immunologically privileged site with the tissue being sequestered

during the development allowing only a few cells to reach the brain through a barrier : the blood brain barrier which controls the passage of fluids and cells into and out of the brain (Moser et al, 1976; Lisak and Zweiman, 1977; De Micco et al, 1986).

1.2.2. Blood Brain Barrier

The neural tissues are surrounded by a series of functional barriers regulating the entry of fluids, electrolytes, small molecules and protein from the blood into the extravascular space (Bradbury, 1979)(See Diagram 4.).

The passage of the above components into the brain takes place by a variety of methods including diffusion, active-transport, and pinocytosis. The rate of entry is regulated by the cerebral capillary cells and the glial cells. Neural tissue is very closely packed together, being derived from ectoderm, and the cells are like the epidermal layer of the skin forcing fluid to pass through rather than around the cells. The brain and spinal cord are bathed in cerebral spinal fluid (CSF) which is derived from the plasma . It has a very low concentration of protein which is prevented from entering by the tight junctions of the cuboidal cells forming the choroidal epithelium and the endothelium of the cerebral arteries. The arachnoidal cells prevent proteins from diffusing into the CSF from the outer layers of the arachnoid. Even with all these barriers there are still discrete areas of the brain where protein molecules and cells can enter more easily.

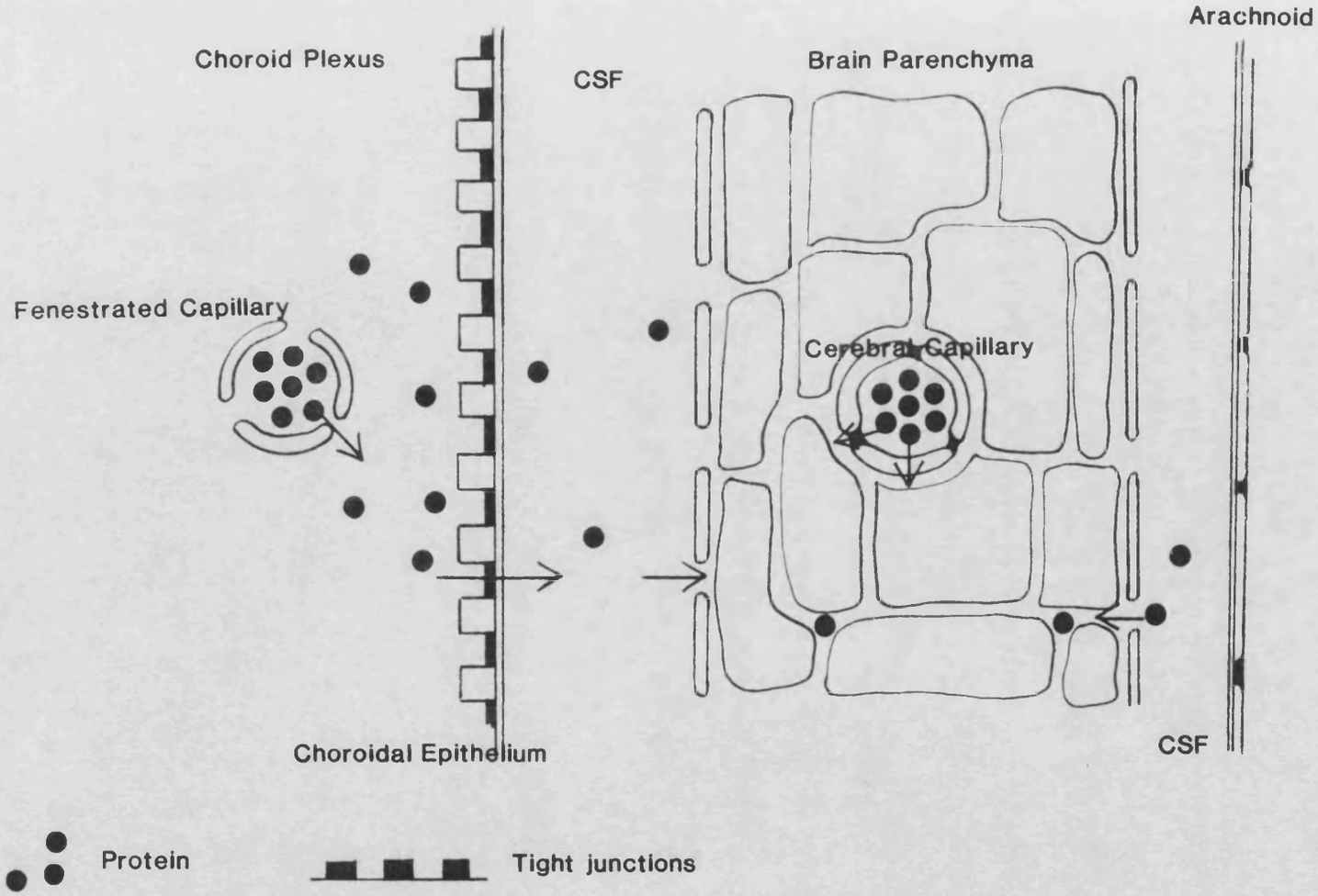


FIGURE 4 The Blood Brain Barrier

The isolation of the brain from all other tissues can be easily demonstrated by injecting trypan blue or Evan's blue intravenously. The dye soon equlibriates and diffuses out of the blood into surrounding tissue. If the tissue is then examined for dye the only tissues not stained are those of the brain and spinal cord. A similar experiment can be repeated by injecting the dye into the brain when only the brain and spinal cord are stained (Leibowitz and Hughes, 1983).

(c) The local immune response in the CNS

Immunoglobulin enters the CNS by diffusion depending on the levels of immunoglobulin in the serum. IgG and IgA occur in low but measurable amounts, whereas only traces of the other immunoglobulins are present. The levels are low compared to those in the serum. Small molecules are able to pass into the brain more easily which explains why IgM, with a high molecular weight, is only present in trace amounts in the CSF. Albumin, a relatively low molecular weight protein, is the most abundant protein in the CSF and can be used to measure how intact the blood brain barrier is.

Complement components C1-C4 are present but only in very low amounts, whereas the C5-C9 have not as yet been detected in the CNS (Bradbury, 1979).

The brain is partially isolated from the normal lymphatic system of the body, has no organised lymphoid tissue of its own

and is without lymph nodes (Darling et al, 1981). There are permanent populations within the CNS of native immunocompetent cells including macrophages (Circolo et al, 1982), T and B lymphocytes (Traugott et al, 1981; Kreth et al, 1982) and haemopoietic stem cells (Bartlett et al, 1982) capable of initiating an immune response. Accordingly only a few B and T lymphocytes in the CNS.

Inflammation can cause any of the barriers previously described to break down (Vandenbark et al, 1985), allowing plasma proteins, immunoglobulins and complement components into the CNS. The increase in cerebral vascular permeability can be shown by measuring the albumin in the CSF and the serum. If the ratio of the two is raised then this indicate some breakdown of the blood brain barrier.

As part of the inflammatory process B and T lymphocytes enter the CNS and immunoglobulin is synthesised intrathecally. Local production of antibody is most pronounced in chronic inflammatory disorders such as syphilis. An increase in CSF IgG levels compared with other plasma proteins is associated with this local production.

SECTION 2 AUTOIMMUNITY

Section 2 Autoimmunity

2.1. Definition

Autoimmunity is that state where the immunological response is directed at the organism's own antigens. It may be transient and reversible or persistent and life threatening. There is an entire area in clinical medicine concerned with illnesses in which autoimmunity plays a dominant pathogenic role; in many of these the detailed pathogenesis and etiology have not been eludicated. There are autoimmune diseases which are mediated by attack on a target organ and are known as organ specific. An autoimmune disease where the effect is widespread is known as a non-organ specific disease.

Normally the immune system reacts against foreign antigens without attacking 'self' components. Such self tolerance is essential for health and when it breaks down autoimmune diseases occur (Ehrlich, 1900). However, the immune system displays a complexity, which includes a requirement for self recognition. Internal regulation involves certain cell-cell interactions which do not occur efficiently unless there is a recognition by one cell of self determinants on the other. Aurameas et al (1984) demonstrated the abundance of autoantibodies in serum of normal individuals. Autoantibodies are consistent with health (Holmberg et al, 1984; Prabhaker et al, 1984; Dighiero et al, 1985) yet their uncontrolled production may lead to disease.

The original hypothesis of Burnet and Fenner (1959) that all anti-self lymphocytes are eliminated before being allowed to mature, is now not acceptable because anti-self B-cells are present in adults. Yet the immune system regulates the production of autoantibodies and remains unresponsive to self-antigens (tolerant). There are three main possibilities of how this tolerance is achieved. The first of these involves clonal elimination during the development of the immune system. During their differentiation B and T cells go through a phase where contact with antigen (self or foreign) induces tolerance. In some way the cells receive a signal which makes them tolerant, either by elimination or by causing functional inactivation without cell death (Roitt et al, 1985) suggest the deletion of anti-self T helper cells making the B-cell clones unable to respond to antigen. Lymphocytes are rapidly replaced so this form of tolerance must be an active continual process (Ryan et al, 1984).

The second mechanism involves compartmentation and exclusion from the immune system. Certain antigens such as lens proteins, brain antigens and spermatazoa become sequestered in avascular sites during development, making them antigenic to the host. Their release would then induce autoantibody formation. Yet many autoantibodies are found in normal individuals and they are often not destructive. If the sequestation theory is correct, then lymphocytes would have to be under some form of active continuous control (Teale et al, 1980; Heshmati et al, 1984)

The third idea is that of active suppression which proposes the continuous suppression of the T helper and B-cell functions by the enhancement of T suppressor cells. This role of T suppressor cells is still unproven (Green et al, 1983).

Autoimmunity defines a state where self tolerance terminates, and the disease occurs as a result of antibodies or cells reacting with self constituents. There are three major effector mechanisms which induce tissue damage, more than one of which can operate in one individual with the disease. This helps to explain the variability amongst patients with the same syndrome even those who are closely related. The tissue damage is mediated either by the action of autoantibodies, the action of immune complexes or by sensitized T lymphocytes (Senitzer et al, 1984).

Autoantibodies cause destruction to tissues by binding to the cell surface and initiating complement activation and lysis. Myasthenic sera cause complement mediated cell lysis to cultured muscle cells.(Childs et al, 1985). However damage can also occur as a result of antibody dependent cell-mediated cytotoxity, when cells with a cytotoxic potential and possessing Fc receptors for immunoglobulin bind and lyse target cells already coated with antibody. This happens in Multiple sclerosis (Baumhefner et al, 1985). In addition to these mechanisms, antibodies can induce disease by binding to functional receptors so inhibiting (e.g. pernicious anaemia), blocking (myasthenia gravis) or

stimulating (Grave's disease) functions without actually killing the cells .

Another factor to cause damage is the formation of immune complexes which are deposited in many tissues of the body. These complexes are believed to be formed in the circulation or intercellular fluids being usually deposited in tissues with large filtering membranes. The kidney, skin, brain and joints are therefore most affected by the damage which usually arises from complement, and the attraction of macrophages and granulocytes (Koffler et al, 1982).

Cell-mediated immunity also gives rise to tissue damage through cell-mediated cytotoxcity. This involves many cell types which, when they become closely bound to the target cell, give "the kiss of death" ie they lyse the target cell. Such cells are usually T-cells and are known as cytotoxic T-cells. Cytotoxic T-cells bind to the target cell via the major histocompatability antigen; a specific antigen but no antibody is required. After binding, the membrane permeability of the target cell is altered, followed by swelling, disruption and lysis. The T-cell survives to go and lyse more cells. In autoimmunity, cytotoxic T-cells are sensitised to self antigens and either cause tissue lesion themselves, attract macrophages via lymphokines or secrete destructive lymphokines. In multiple sclerosis (MS), the cytotoxic T-cells outnumber T helper cells by 5:1 in the cerebral spinal fluid compared to healthy controls. There is also

migration of the T helper cell from the peripheral blood into the CNS of MS patients where further destruction occurs (Booss et al, 1983).

Autoimmunity develops in individuals either through a genetic defect or a defect in the immune system. There is undoubted familial incidence of autoimmunity e.g. thyroid autoantibodies are linked with abnormalities of the X chromosome. These genetically linked autoimmune diseases are more often specific for one organ, (e.g. Hashimoto's disease) and patients' relatives often have a high titre of thyroid autoantibodies (Roitt et al, 1985). Further evidence for the operation of genetic factors in autoimmune diseases is given by the fact that they tend to show associations with particular HLA specificities (e,g B8, Dr4 in Rheumatoid arthritis, B8, Dr3 in Myasthenia Gravis and Addison's disease)(Harrison and Behan, 1986).

Immunological control mechanisms operate in the normal individuals who possess both self-reactive lymphocytes and autoantibodies. These must be suppressed to prevent autoimmunity occurring. Autoimmunity may arise by direct activation of T helper cells or activation of another cell which renders T helper cells resistant to suppression. Autoantigens could also by-pass the T helper cells to directly stimulate the T effectors and B-cells. Also defects in antigen specificity can inactivate T suppressor cells, non-specific T suppressors or idiotypic T suppressors.

Some clones of self reactive B-cells show an innate ability to respond without the requirement for T-cell help and the primary defect may lie in these cells. B-cells can be triggered directly to produce autoantibodies by polyclonal activators such as Epstein Barr virus or bacterial polysaccharides. These autoantibodies are however only of the weakly binding IgM class which cause little damage so are unlikely to be of primary importance. However in genetically predisposed hosts they may act as accelerators of the disease (Jyonovchi et al, 1981).

Also cross-reactivity of exogenous agents with host antigens can lead to autoimmunity. A severe infection with hemolytic streptococcus which causes acute rheumatic fever can often elicit an immune response where the antibodies cross react with self cardiac muscle antigens (Kaplan et al, 1964).

2.2. Examples of Autoimmune Diseases

2.2.1. Myasthenia Gravis

Myasthenia Gravis (MG) is an organ specific autoimmune disease affecting predominantly young women. The disease is characterised by extreme muscle weakness associated with antibodies to the acetylcholine receptor (ACHR) present on the surface of the muscle membranes. The ACHRs are localised at the motor end plate where the nerve impulse is transmitted by the release of acetylcholine from the nerve terminal to the muscle. In MG the ability to synthesise or release acetylcholine is not affected. Examination of the motor end plates by immunocytochemical techniques demonstrates IgG, C3 and C9 deposited on the post-synaptic folds of the muscle (Sahashi et al, 1980). Few cells infiltrate the MG end plates, so effector cells are rarely involved. The means by which loss of ACHR occurs is still unclear but the anti-ACHR antibody probably consitutes the primary agent. Autoantibodies could act via one or more of three mechanisms, namely: the direct blockade of the receptor by antibody; complement-mediated lysis of the post synaptic membrane and the accelerated degradation of the post-synaptic membrane (antigenic modulation).

Workers have sought for abnormalities in the cellular immune system in MG (Lisak et al, 1982; 1985) For the regulation of the immune response, normal functioning of distinct lymphocyte

2.2. Examples of Autoimmune Diseases

2.2.1. Myasthenia Gravis

Myasthenia Gravis (MG) is an organ specific autoimmune disease affecting predominantly young women. The disease is characterised by extreme muscle weakness associated with antibodies to the acetylcholine receptor (ACHR) present on the surface of the muscle membranes. The ACHRs are localised at the motor end plate where the nerve impulse is transmitted by the release of acetylcholine from the nerve terminal to the muscle. In MG the ability to synthesise or release acetylcholine is not affected. Examination of the motor end plates by immunocytochemical techniques demonstrates IgG, C3 and C9 deposited on the post-synaptic folds of the muscle (Sahashi et al, 1980). Few cells infiltrate the MG end plates, so effector cells are rarely involved. The means by which loss of ACHR occurs is still unclear but the anti-ACHR antibody probably consitutes the primary agent. Autoantibodies could act via one or more of three mechanisms, namely: the direct blockade of the receptor by antibody; complement-mediated lysis of the post synaptic membrane and the accelerated degradation of the post-synaptic membrane (antigenic modulation).

Workers have sought for abnormalities in the cellular immune system in MG (Lisak et al, 1982; 1985) For the regulation of the immune response, normal functioning of distinct lymphocyte

subsets is necessary. The total number of B and T lymphocytes reported in MG compared to normal controls have been determined by many groups of workers with no clear conclusions. The overall indications are of increased B-cells and decreased T-cell populations in both the thymus and peripheral blood (Harrison and Behan, 1986).

An idea of Miller and Schwartz (1982) that defective suppressor T-cell activity could result in the elevated levels of anti-acetylcholine receptor antibodies found in MG was supported by demonstrations in such patients of elevated levels of other autoantibodies (Huang et al, 1981; Limburg et al,1983; Smith et al, 1984). However, if one removes the suppressor T-cell population from normal individuals, anti-acetylcholine receptor antibodies are not synthesised (Lisak et al, 1984). The relative proportions of T-cell helper and T-cell suppressor populations of MG patients have been compared to normal controls. The results are conflicting and inconclusive (Harrison and Behan, 1986).

Studies of the in vitro responses of peripheral blood lymphocytes to non-specific mitogens have been shown to be normal (Housley and Oppenheim, 1967; Abdou et al, 1974; Tronconi et al, 1979; Wijermans et al, 1980; Trotter et al, 1982) and impaired (Simpson et al, 1976; Huang et al, 1977; Zilko et al, 1979; Sagar et al, 1980; Berrih et al, 1981; Koethe et al, 1981; Dropcho et al, 1982) in MG patients. However the proliferative response to acetyl choline receptor has been consistently reported to be

increased (Harrison and Behan, 1986).

Multiple Sclerosis

Multiple Sclerosis (MS) usually occurs in young adult life with symptoms developing over a period of years. The characteristic lesions of MS are sharply demarcated plaques of demyelination in the CNS (Lumsden, 1972). They are present in the brainstem, cerebellum, spinal cord and optic nerves. In histological sections stained for myelin the sheath is seen to cease abruptly at the margin of the plaque, the neurones and axis cylinders survive but may disappear in time.

The most widely held theory about the aetiology and pathogenesis of MS invokes autoimmunity (Leibowitz, 1983).

Blood lymphocyte numbers have been found to be normal in MS (Weiner et al, 1979; Hauser et al, 1981; Brinkman et al, 1982) yet there are a few reports of reduced numbers (Reddy et al, 1976; Sagar et al, 1979). The lymphocyte count does not seem to vary with MS disease activity (Hauser et al, 1981) or duration (Brinkman etal, 1982). The majority of data indicate that the percentage of T lymphocytes in the blood of MS patients is not significantly different from normal controls or patients with other neurological disease, although a number of studies report a decrease in T-cell number in the blood of MS patients when the disease is clinically active. T-cell suppressor activity has been reported to be defective when the disease is active and normal when the disease is stable (Antel, 1978; Huddleson and Bloom, 1979). However a concensus on the results of lymphocyte sub-

1979). However a concensus on the results of lymphocyte sub-populations in MS as compared to normal controls has not been achieved by different groups of workers. Several studies showed an elevated number of B-cells especially with a decrease in T-cells (Reddy et al, 1976; Lisak et al, 1975; Schauf et al ,1977; Sagar et al, 1979). There is one report however of a decreased number of B-cells in the blood of MS patients (Kam-Hansen et al, 1978). T suppressor cells have been reported to increase with exacerbations (Antel et al ,1978; Antel et al, 1979).

In a number of reports MS blood lymphocyte proliferative responses to various mitogens were found not to differ from those of normal controls (Weiner et al, 1979; Goust et al, 1980; Schauf et al, 1981;Brinkman et al, 1982; Haahr et al, 1983;) or of patients with other neurological diseases (Hughes et al, 1977; Brinkman et al, 1982). Reactions to phytohaemagglutinin (Hughes et al, 1977; Weiner et al, 1979; Goust et al, 1980; Schauf et al, 1981), Concanavalin A (Knight et al, 1975; Weiner et al, 1978; Sheremata et al, 1979) and Pokeweed mitogen (Knight et al, 1975; Weiner et al, 1978; Goust et al, 1980; Brinkman et al, 1982) were all investigated. Some studies however have shown a decrease in response to several mitogens in MS (Antel et al, 1978; Nordal et al, 1978; Dropcho et al, 1979; Walker et al, 1979; Ilonen et al, 1981).

The percentage of T-cells increases and the percentage of B-cells decreases in the CNS of MS patients but this is generally seen in patients with a variety of neurological diseases. Both

significant depressions of T suppressor cells during active disease followed by a significant increase in these cells during recovery have been recorded in the CSF. (Huddleston and Bloom, 1979)

Antimyelin antibodies have been demonstrated in MS (Lisak et al, 1975; Wolfgram et al, 1976). There is a considerable evidence that demyelination in MS is immunologically mediated (Lisak, 1980; Raine, 1984) but the target antigen is still unknown. The myelin-associated glycoprotein (MAG) has been found to be preferentially depleted in myelinating plaques. However the detection of anti-MAG antibodies has not been successful in the blood by RIA (Nobile-Orazio, 1985) or the CSF (Nobile-Orazio, 1986) of MS patients.

The recent report by Koprowski et al (1985) that human T-cell lymphotrophic retroviruses (HTLV) may be involved in the development of MS has aroused interest. A study by Hauser et al (1986) investigated the presence of HTLV-like sequences in tissues from the CNS of MS patients and Karpas et al (1986) investigated the presence of antibodies against these retroviruses in the serum amd CSF of MS patients with no success. On the whole the results are very contradictory with no clear idea as to the cause of the disease.

SECTION 3 MOTOR NEURONE DISEASE

Section 3. Motor Neurone Disease

3.1. Introduction

The clinical components of motor neurone disease (MND) were described over a century ago by Aran (1850), Duchenne (1860), and Charcot (1869). This fatally progressive neurological disorder of adult life is characterised by clinical evidence of widespread damage to motor neurones, resulting in wasting, weakness, and fasciculation of skeletal muscles. Almost invariably pyramidal tract signs appear at some stage of the illness. The clinical features are attributable to the degeneration of the motor neurones at the spinal and brain stem levels, or at both levels, usually with some involvement of the Betz cells of the cortex and demyelination of the corticobulbar and corticospinal (pyramidal) tracts.

Several variants of the disease are recognised, progressive muscular atrophy (Aran, 1850) occurs when the disease is confined to the anterior horn cells of the spinal cord where the limb motor neurones are predominantly affected; progressive bulbar palsy (Duchenne, 1860) when the cranial motor nuclei are involved; and amyotrophic lateral sclerosis (Charcot, 1869) when there is pyrimidal tract involvement causing degeneration of the corticospinal pathway. The clinical differentiations are indistinct and it is common in a clinical series to class them as a variants of one disorder ~ motor neurone disease (Friedman et al,1950; Mulder,1957; Mackay,1963; Bonduelle et al,1970). However

in the United States, the generic name amyotrophic lateral sclerosis is preferred to motor neurone disease.

The diagnosis of MND is sometimes difficult at the early stages of the disease. This is so because of the similarity of symptoms to those of other neurological disorders. In general, diagnosis is based on electro-myography which involves measuring the electrical activity of muscles using microelectrodes. Muscles which have lost their nerve supply will have impaired response to stimuli.

MND is usually a fatal condition with death occuring within 3-5 years of onset (Clifford-Rose, 1984) although 20 per cent of patients survive upto 5 years and 10 per cent survive up to 10 years (Mulder and Howard,1976). It is an uncommon but ubiquitous disease with a prevalence of 2.7-7 per 100,000 population (Kurland et al,1958,1969), although pockets of high prevalence exist in certain tribal communities of the Western Pacific Islands, especially on Guam. The Chamorro people of Guam have an annual incidence rate of 32.3 per 100,000 (Brody et al,1972).

The mean age of onset is about 52 years, with 98 per cent between the age range 35-84 years. A remarkable feature of all types of motor neurone diseases is that, throughout the world, the male rate is from 1.2-2.5 times higher than the female rate. A familial form has a 1:1 male to female ratio, as is usual with autosomal traits (Hawkes et al, 1984). There are no well documented racial differences for the susceptibility of MND, with

the exceptions of Guam and Japan. However, there are reports of a slight increase in rate among Japanese in the United States and a suggestion of an elevated rate among Filipinos in Hawaii (Matsumoto et al, 1972).

Despite the apparent simplicity of the pathological process in MND there is an almost complete lack of knowledge of its cause, or of possible therapies.

3.2. Aetiology

Many possible factors have been considered as being involved in the aetiology of MND. Some of these will be briefly discussed here.

3.2.1. Carbohydrate Metabolism

A number of studies have shown impaired glucose metabolism in patients with ALS, (Gotoh et al, 1972; Gustafson and Stortebecker, 1972; Koerner, 1976; Nagano et al, 1979). Recently Murai et al (1983) confirmed that serum glucose levels were significantly higher in MND than in healthy controls although the insulin response to glucose loading was normal. These results contradict those of Gotoh et al, (1972) and Nagano et al, (1979) who found impaired pancreatic secretion of insulin which may result in the abnormal glucose metabolism.

It is now generally accepted that glucose intolerance is a common finding in MND, although the pathophysiological mechanisms involved in the carbohydrate abnormality are still disputed.

3.2.2. Lipoprotein Metabolism

Only a few reports have been published on lipid and lipoprotein metabolism in MND. Mueller et al, (1970) showed significantly elevated fasting concentrations of serum free fatty acids and cholesterol while Gustafson and Stortebecker (1972) found an increased incidence of type II hyperlipoproteinemia in ALS. Huang et al, (1978) found an abnormal composition of high density

lipoprotein (HDL) with reduced levels in familial ataxia and Friedeich's ataxia. Recently, Murai et al, (1983) found lowered HDL cholesterol levels and increased triglyceride levels in ALS patients as compared with healthy controls.

In patients with MND, skeletal muscle fibres are progressively denervated resulting in striking changes in their metabolic capabilities. It may be that the abnormalities observed in denervation have no particular pathogenic significance for MND but rather result from general metabolic derangement.

3.2.3. DNA

It has been suggested that certain neurological disorders arise from abnormalities in DNA and its function (Bradley et al, 1982; Mims et al, 1983). Such defects may occur in several ways e.g., as an error in the DNA code during semi conservative replication or a defect in the DNA repair mechanism. Neurones are in post mitotic phase and mutation may not be corrected because of the low repair activity (Scudiero et al, 1976). Neurones may even be more prone to mutations due to virus infection (Doolittle et al,1984). Severe damage to motor neurone nuceli is commonly observed in MND. Mann and Yates (1974) found shrinkage of the nucleus and hyperchromatic condensation of the nuclear chromatin followed by loss of basophilia of the nucleolus and a reduction of the cytoplasmic RNA content . Davidson and Hartmann (1981)

found a 30-40% reduction in RNA content of patients with ALS, and they presented evidence to suggest that it was restricted to the motor system. This reduced RNA content was not a function of age or cellular loss. The base composition of RNA from motor neurones of ALS patients differed in that it had a low percentage of adenine, and a low adenine to uridine ratio. Auto-antibodies to single-stranded and double-stranded DNA are elevated in some autoimmune disorders and infectious diseases (Tang et al, 1979). The presence of DNA in circulating immune complexes in MND sera was recently investigated (Unger et al,1985). The ratio of single-stranded : double-stranded DNA was found to be significantly increased with respect to normal controls. Bradley et al (1982) suggested that ALS is due to a deficiency in the normal DNA repair mechanisms with resultant accumulation of damaged DNA, which causes the abnormal transcription of RNA and thus translation of abnormal protein or absence of synthesis of specific proteins.

3.2.4. Mineral and Metal Metabolism

Calcium metabolism in ALS was found to be abnormal in approximatly 20 per cent of cases compared with normal controls. The patients were found to be hypocalcaemic with elevated levels of parathyroid hormone (PTH), indicating secondary hyperparathyroidism (Patten, 1984).

Plasma levels of 25-hydroxy-vitamin D were found to be low

in ALS patients with abnormal calcium metabolism. This mild vitamin D deficency, could have caused the hypocalcaemia, as calcium metabolism was returned to normal by administering a vitamin D analogue. However, even though calcium metabolism was corrected there was no reinnervation of the motor neurones (Patten, 1984).

There is a significantly increased incidence of congenital abnormalities in ALS (Campbell et al, 1970). The relationship between calcium metabolism, and vertebral structure in MND is unclear.

Yase (1980) noted that when there is severe calcium deficiency calcium in the form of hydroxyapatites is deposited in soft tissues, including the brain. These may be caused by secondary hyperparathyroidism in infancy and childhood (chronic deficiency). The accumulation of the hydroxyapatites in motor neurones could eventually destroy the cells (Yase, 1968; 1973; 1979;1980) as in MND. Long term calcium deficiency also affects metal toxicity rendering metals such as aluminium, iron, and manganese toxic because of increased absorption from the gut, decreased renal excretion and decreased bone deposition of these metals in bone. Instead the metals are deposited in soft tissues such as the brain where they are a poison becoming chronically toxic to the motor neurones and are thought to lead to the premature appearance of neurofibrillary tangles seen in ALS and Parkinson's Dementia (Gajusek, 1984).

Patten (1984) found that exposure to lead and mercury are antecedent events in motor neurone disease. Lead has several types of toxic effects on the nervous system (Simpson et al, 1964). Lead intoxicated patients have been documented with weakness and wasting of muscles with some pyramidal tract disease and fasciculations (Wilson, 1907) thus mimicking MND. Lead levels in plasma (Conradi et al, 1978) and cerebral spinal fluid (Conradi et al, 1980) were increased in MND, compared to normal controls, and increased levels of lead in tissues, including the anterior horns, have been reported by Kurlander et al (1979). There are also reports of high incidences of exposure of ALS and MND patients to lead before disease onset (Felmus et al, 1976; Pierce-Ruhland et al, 1981). Lead exposure usually results in lead storage in bone, the major mechanism of detoxification (Barry, 1975). Demineralisation of the skeleton in aging, fracture, cancer, or serious trauma or infection can result in the transfer of lead from bone to soft tissues where it is a poison. This could explain the increased incidence of fractures (Felmus et al, 1976; Kurtzke et al, 1980), bone disease (Campbell, 1970; Patten, 1982), trauma (Kurtzke, 1980) and cancer in MND patients.

Chelating agents are used to mobilise lead from the nervous system of patients with MND preventing further damage. Remission of ALS can result, and this remains the only real evidence for the toxic origin of MND (Patten, 1984).

3.2.5. Enzyme Defects

MND results in damage to the neuromuscular system leading to eventual failure. Nerves contact muscle at the muscle end plates, and these structures control the information transfer from the muscle to the nerve and vice versa. Acetylcholinesterase (AChE) is an essential consituent of the end plate and contains a collagen-like tail which may interact with fibronectin. It was suggested by Festoff et al (1980) that the continuous degeneration of the motor system results from the steady decrease in adherence of AChE to the basement membrane because of the activation of a latent collagenase which allows collagen to be resorbed.

3.2.6. Neurotransmitters and aminoacids

Spinal motor neurones are metabolically highly active and are subject to a number of neural influences from the descending cortical and brain stem tracts, as well as from segmental spinal afferents and local inhibitor and excitatory interneurones, such as Renshaw cells (Belleroche et al, 1984). The excitory transmitters, aspartate and glutamate, and the inhibitory amino acids glycine, taurine, and gamma aminobutyric acid (GABA) have a direct effect on spinal motor neurones (Davidson, 1976). Many other transmitters are present and could possibly contribute to the regulation of the motor neurone activity e.g. Noradrenalin, dopamine, enkephalin, 5-hydroxytryptamine and substance P

(Belleroche et al, 1984).

Abnormalities in the metabolism of these neurotransmitters may contribute to the mechanism of the disease. Studies of neurotransmitters in MND are of two types. Firstly there are those dealing with the measurement of levels, and changes in their rates of biosynthesis and degradation. Secondly, those concerned with measurement of neurotransmitter receptors. Biochemical analysis is usually carried out on serum and CSF samples, and spinal cord and brain post mortem tissue.

Yoshino et al (1976) found diverse changes in amino acids in the ALS spinal cord, with an increase in the taurine content. They also found (Yoshino et al, 1979) seven amino acids that showed statistically significant changes, (taurine was the only one which increased constantly) in the motor cortex of ALS patients compared with normals.

Patten and coworkers (1978) found elevated levels of amino acids in the CSF and serum of MND patients compared with neurological controls and observed an increase in the levels of ammonia and ornithine in anterior horn spinal cord tissue of ALS patients (Patten et al, 1982). Belleroche et al (1984) measured the levels of a number of amino acids in the CSF of MND and age matched controls and showed that marked abnormalities do occur. Large increases (upto 60%) were observed among both essential and non-essential amino acids. The most marked increase was in the level of glycine. The changes in amino acid levels, particularly in glycine, alanine and taurine showed correlations with disease

severity and duration.

In summary, a number of amino acids are significantly elevated in MND when compared with age matched controls. However, it is not clear yet whether this results from enhanced catabolism and\or reduced efficiency of amino acid uptake from the CSF.

3.2.7. Receptor Defect

Androgen receptors have been demonstrated in both the cranial nerve, and spinal motor neurones (Sar and Stumpf, 1977). However, cranial nerves III, IV, and VI and the dorsal root motor nuclei of cranial nerve X have very few androgen receptors, and these structures are invariably spared in motor neurone disease. Weiner (1980) suggested that MND may be a disease where androgen receptors are lost from motor neurones or are not functioning. In ageing men oestrogen levels increase and testosterone levels decrease possibly because testosterone binding globulin removes free testosterone from the circulation. In normal people axons can be repaired with the anabolic help of androgens. However, in MND , androgen receptors are lost and the axonal changes result in the death of the motor neurone (Weiner, 1980).

Hayashi et al (1981) reported that compared to normal spinal cord taken from ALS patients showed a significant loss in the number of glycine receptors as measured by ^{3}H-strychnine binding to the anterior grey areas of the spinal cord taken from ALS patients compared to controls. Whitehouse et al (1983) found that muscarinic, cholinergic, benzodiazepine, and glycine receptor concentrations were all diminished in cervical, thoracic, and

lumber spinal cords of MND patients, and that the distribution of these receptors within MND spinal cord differed from normal.

Recently, infusions of thyrotropin-releasing hormone (TRH) have been claimed to alleviate the weakness of MND patients (Engel et al, 1983). Receptors for TRH are present on motor neurones and their density and distribution in the MND spinal cord have been investigated. Reduced density and altered distribution of TRH receptors in MND spinal cord have been found (Mitsuma et al,1984; Manaker et al, 1985).

The reduction in receptor distribution can be explained by destruction and loss of motor neurones in MND. However, the altered pattern of distribution particularly in those areas of the spinal cord where motor neurones are not damaged could suggest some kind of defect in receptors.

3.2.8. Genetic Defects

Motor neurone disease can be inherited within families, in which case it is called familial MND. Inheritance is mainly autosomal dominant (Engel et al, 1959) and gives rise to a progressive illness affecting predominantly the lower motor neurones leading to death within an average of 4 to 7 months. Post mortem studies reveal severe cell loss in the anterior horns and occasional degeneration of the pyramidal tract. The mean age of onset is 45.8 years with nearly equal sex incidence. According to Alter and Schaumann (1976) familial cases comprise 2-15 per cent of motor neurone diseases.

Histocompatibility antigens have been measured for the A, B, and C loci in various populations of MND. Antel et al (1976) reported an increased incidence of HLA-A3 in a group of 44 patients when compared to 200 controls. Terasaki and Mickey (1975) found the overall incidence of HLA-A3 in ALS was also increased but not significantly. However, Pederson et al (1976) found no such changes. Hoffman et al (1977) found an increase in Bw-16 in Guamanian patients but it was not significant. Hoffman et al (1978) reported that cell mediated immunity was decreased, and that all the hyporesponsive patients had Bw-35. Behan et al (1976) only found an increased incidence when HLA-A2 and A28 were combined . Kott et al (1976) found cellular immunity to polio virus in two thirds of patients with ALS, with an increased incidence of A3 and Bw 35. Most recently Bartfeld et al (1982) found a slight increase in HLA-B17 and B8 and a slight decrease in the frequency of HLA-A9 in patients with ALS. Knott et al (1976) reported that HLA-Bw 35 was increased in Israeli patients. Bartfeld et al (1982) analysed their results taking the report by Kott et al (1982) into consideration, and separated the patients by race. They found that non-Jewish patients with ALS had a significant increase in Bw 35 compared with controls. Zaiwalla et al (1984) on the other hand reported no relationship between HL antigen and MND.

3.2.9. Polio Virus

Polio virus has the capacity to produce paralysis in the absence of other neurological signs and has prompted speculation that it may be a causative factor in MND. Polio virus has been implicated in virtually all types of motor neurone disease.The most commonly reported syndrome has been poliomyelitis followed by delayed muscular atrophy. There are now over a hundred cases reported in the literature (Campbell et al, 1969; Mulder et al, 1972) most of which are males who experienced severly disabling poliomyelitis in early childhood and then neurological improvement and stabilisation.In general paralysis begins after 35-40 years, and lacks any pyramidal tract signs. In 1934 Alajouanine described a woman (40 y) who had a lower motor neurone disorder having had polio. There was notable chromatolysis of the motor neurones. Fragments of her brain stem were injected intracerebrally into a rabbit which developed hind and then fore limb paralysis, resulting in death within six months.Pathological findings in the rabbit closely resembled those of the patient.

Zilkha (1962), Poskanzer et al (1969), and Norris (1975) all reported an increased incidence of prior poliomyelitis in patients with classical MND.

There have been many investigations, virological and immunological, seeking evidence to support the involvement of polio virus in MND. Humoral immunity to polio has been sought in MND patients by measuring neutralising or complement-fixing anti

polio titres without success (Cramer et al, 1973; Lehrich et al, 1974; Jokelainen et al, 1977; Kurent et al, 1979; Kascsak et al, 1982). However the possibility of an antibody-mediated abnormality in MND has been supported by reports of immune complexes in tissue sections of kidney (Oldstone et al, 1976), skin (Palo et al, 1978), and jejunum (Pertschuk et al, 1979), but without evidence of polio antigens.

A variety of techniques including, cell fusion, indirect immunofluorescence for viral antigens, electron microscopy, and haemadsorption has failed to recover polio virus from tissues of ALS patients (Miller et al, 1980).

It has also been postulated that, like retroviruses, polio virus inserts non-infectious RNA into host cells. The RNA retains the ability to produce injurious proteins or defective particles but remains undetectable by conventional infectivity studies. Roos et al (1980) and Miller et al (1980) have looked at molecular hybridisation (The DNA from MND patients is hybridised with polio virus RNA), but no polio virus has been detected.

Kott (1976) reported that in vitro cultures of MND lymphocytes showed production of a migration inhibition factor after challenge with polio vaccine in ALS. Studies of cellular immunity in classical and post polio motor neurone diseased patients by Behan (1977) however found no abnormalities in response to skin test challenge with polio virus although he did observe reduced responses of T lymphocytes to PHA in both groups

of patients compared with normals and normals with a prior history of polio.

As already mentioned HLA-A3 was found to be abnormally prevalent (Antel et al, 1976; Kott et al, 1979) in MND. HLA-A3 and A7 are reported to be increased in polio patients (Pietsch et al, 1973), but this has not been confirmed. HLA antigens serve as genetic markers for immune responsiveness, particularly in viral and autoallergic diseases. It has been suggested that MND is a persistant slow viral infection thus leading to the study of HLA antigens.

Virally-initiated immunological attack on motor neurones could start in a number of ways. An obvious possibility is that of fortuitous cross-reactivity between a viral antigen and one on the motor neurone membrane. Alternatively anti-idiotypic antibodies to anti-viral antibodies might interact with virus receptor sites on neuronal cells.

However, evidence implicating polio virus in the pathogenesis of MND is inconclusive.

3.2.10. Immunological Involvement in MND

Motor neurone disease is a highly specific disease causing degeneration only of the motor neurones. This specificity has the nature of an antigen-antibody reaction, and an autoimmune disorder. This possibility has been debated for years without any clear evidence which could prove or disprove the idea.

3.2.10.(a) Immune Complexes

The usual laboratory indices of abnormality in the immune system, serum immunoglobulin and complement levels, and peripheral white blood cell counts have been reported to be normal (Whitaker, 1973; Behan, 1979; Antel et al, 1982). The CSF is usually normal in these respects although electrophoretic studies show minor alterations in protein levels, consistent with blood brain damage. Increased concentrations of immunoglobulin in the CSF have been reported (Behan, 1979; Leonardi etal, 1984). Positive evidence in favour of an immune system involvement in MND is provided by the detection of immune complexes in renal tissues (Oldstone et al, 1976; Norris, 1979), spinal cord and motor cortex (Donnenfeld et al, 1984) and of elevated levels of such complexes in sera (Hoffman et al, 1978). A more recent study by Bartfeld et al (1982) found an increase in serum immune complexes over normals but no greater than in neurological controls. In these studies, the circulating immune complexes were determined by C1q binding assays. The Raji cell binding assay has led to reports of both slightly elevated (Antel et al, 1979) and normal (Tachousky et al, 1976) levels of immune complexes in MND sera. Characterisation of DNA in circulating immune complexes in MND sera showed a significantly higher ratio of single -stranded : double-stranded DNA with respect to control (Unger et al, 1985).

3.2.10. (b) Cells

Immune cell abnormalities in MND have been sought with mixed success. The cutaneous response to common antigens was found to be decreased in some MND patients (Kott et al, 1976; Hoffman et al, 1978; Behan, 1979). The percentages of immunoregulatory T cells measured using monoclonal antibodies was found to be normal in MND patients (Bartfeld et al, 1985). In vitro responses to non- specific mitogens such as PHA and ConA have been reported to be abnormally low (Hoffman et al, 1978; Behan, 1979). However, Antel et al (1979) and Bartfeld et al (1982) were unable to demonstrate any differences. T-cell counts were found to be normal as were the B-cell counts (Antel et al, 1979). The T-cell subsets measured by the expression of receptor however were significantly different. The Fcμ subset was decreased relative to the Fcγ subset (Westall et al, 1983).

3.2.10. (c) Autoantibodies

Studies of potential autoantibodies in MND have sometimes given positive results, but are rather controversial. Both serum and spinal fluid gammaglobulin levels have been reported to be relatively high in some MND cases compared to normals, but not to neurological controls (Roboz et al, 1969; Tavolate et al, 1975). The high gamma globulin levels may indicate a high incidence of infectious complications in MND patients. Hoffman et al (1981) reported high levels of IgA and IgG in Guamanian MND patients.

Serum samples from patients with MND were used as controls in early studies of myelintoxic antibodies in Multiple sclerosis (MS), and were found to cause demyelination in myelinating cultures just as frequently as the MS sera (Bornstein et al, 1965; Field et al, 1969). The presence of auto antibodies to myelin has also been shown by immunofluoresence (Edgington et al, 1970; Lisak et al, 1975). Schauf et al (1980) reported that serum from MND patients reduces the electrical response from the central roots of a frog spinal cord preparation when the dorsal root was stimulated. The blocking activity seemed to correlate with disease activity and could reflect the presence of a neurally-directed antibody (Schauf et al, 1978). While such serum factors would be interesting in a primary demyelinating disease, in MND the actual relevance is obscure because there is primary neuronal degeneration and only secondary demyelination.

There have been reports of elevated levels of serum antibodies to neurofilament antigens in neurological disorders (Bahymanyar et al, 1983; Bahymanyar et al, 1984; Toh et al, 1985; Jehanli et al, 1986).

An initial report of Wolfgram and Myers (1973) of a serum borne neurotoxic factor in MND has been recently confirmed by Roissen et al (1982) but there have been contradictory reports (Horwich et al, 1974; Liveston et al, 1975; Obatak et al, 1976). However, Digby et al (1982; 1983; 1984; 1985) have reported that MND sera contain immunoglobulins that bind specifically to foetal

rat spinal cord cells in culture. Antibodies in MND sera which bind to antigens present in homogenates of motor neurones and dorsal root ganglia have been recently found (Brown et al, 1984; Kletfi et al, 1984).

Gurney and Apatoff (1984) demonstrated that antisera against components of conditioned medium from cultures of denervated rat diaphragms are capable of suppressing terminal axon sprouting and that such polyclonal sera recognised a 56K protein secreted from muscle into the medium. In addition, it was shown that sera from MND patients block terminal axon sprouting, and also have an antibody against a 56K antigen (Gurney et al, 1984). There are also reports of antibody activity against a protein in the motor neurones (Apatoff et al, 1984) and to a "survival factor" for cultured spinal cord neurones (Kletti et al, 1984). Gurney et al (1984) observed that sera found to supress sprouting in vivo have been shown to block the activity of a muscle-derived survival factor, and have produced a monoclonal antibody that blocks the survival factor for cultured rat spinal cord neurones. Factors from denervated muscle which lead to motor neurone survival and neuritic extension have been reported by others (Eagleson et al, 1983; Slack et al, 1983; Nurscombe et al, 1984), but this report by Gurney et al (1984) has still to be confirmed.

3.2.11. Other Suggested Pathogenic Factors

Many other factors have been reported in association with MND, but usually in small numbers of cases. These include milk ingestion (Patten, 1984), bone fracture (Pierce-Ruland et al, 1981), heavy labour (Breland et al, 1967), pneumatic drilling (Alpers et al, 1949), mechanical injuries (Campbell et al, 1970; Kurtze et al, 1980; Kondo et al, 1981), electrical shock (Haynal et al, 1964), spinal anaesthesia (Ask-Upmark et al, 1961) and various diseases, including malignant neoplasia (Norris et al, 1965).

Environmental factors may have a significant effect on the occurance of ALS. There are three foci of high incidence of ALS in the Western Pacific area. In all three foci a syndrome of Parkinsonism Dementia (PD) and associated Parkinson Syndromes have been seen with a high incidence paralleling ALS. To date, an extensive search for the cause in a slower virus infection (Gibbs et al, 1972), genetic (Reed et al, 1975) or immunological defect (Hoffman et al, 1978; 1981) or a neurotoxic item in the diet (Whiting 1963; 1964; Kurland, 1972) has been unsuccessful. There are recent reports that the incidence of the disease is declining and has been over the past three decades. Garruto et al, 1985 report that the high incidence rates of ALS and PD occuring amoung the Chamorros of Guam have declined to rates only slightly higher than those observed in the continental United States. This decline has occured among males especially those born after 1920

and living in areas where calcium and magnesium levels are low in the soil, and drinking water. The male ratio now approaches unity, compared with the ratio 2:1 in ALS and 3:1 in PD three decades ago. These changes suggest that the high incidence resulted from defects in mineral metabolism, provoked by calcium and magnesium deficiencies, resulting in deposition of calcium and aluminium in neurones. Essentially, however the results indicate very clearly that an evironmental variable has been responsible for the disease (Garruto et al, 1981; Gajudusek, 1984).

SECTION 4 LYMPHOCYTE ACTIVATION

Section 4 Lymphocyte Activation

Lymphocyte proliferation occurs when specific clones of cells reactive against foreign antigens are amplified in vivo (Clonal selection). Cultured B and T lymphocytes can be induced to proliferate in vitro in response to antigens, including those on allogeneic cells (Robbins, 1964). However studies of the proliferative mechanisms are difficult to follow because of the small population of cells that actually respond to specific antigen (Ada, 1970). These problems may be avoided by using polyclonal activators that are able to stimulate lymphocytes irrespective of their antigen specificity.(Wedner and Parker, 1976) 70 - 80 per cent of cells in a given lymphocyte population may be so stimulated (Andersson et al, 1972). The polyclonal activation caused by the non-specific mitogens can sometimes occur in specific lymphoctye subsets. Hence they can be used to study mechanisms of proliferation or immune competence of particular lymphocyte types (Baran,1985).

4.1. Polyclonal Activators

The most commonly used polyclonal mitogens are the lectins, a group of carbohydrate-binding proteins. Lectins bind to the oligosaccharide residues on the cell membrane, and being multivalent, cross link surface molecules as the first step in lymphocyte activation. Lectins were initially characterised by their ability to agglutinate red blood cells, and during these

studies their mitogenic properties were discovered.

Phytohaemagglutinin (PHA) comes from the red kidney bean, Phaseolus vulgaris. The exact concentration of PHA required for lymphocyte activation depends very much on the precise experimental conditions used, but, for human lymphocytes in medium containing 10% (w/v) serum, the best PHA preparations are generally most active when present at 1-5 μg/ml (Ling,1978). PHA transforms a major proportion of the T lymphocyte population. It also appears to affect some B cells, although their response seems to be T-cell dependent (Waxdal & Wilson, 1975). It is thought that PHA-stimulated lymphocytes are preferentially the helper T-cell population (Ramagnani et al, 1977).

Concanavalin A, (Con A) is the best characterised of all the plant lectins. It may account for up to 3% of the protein of the Jackbean (Canavalia ensiformis) and was first obtained in a crystalline form many years ago (Summer 1919). It binds glycoconjugates containing α -D glucopyranosides, and α - D - mannopyranosides, binding specifically with C3, C4 and C6 hydroxyl positions of the pyranoside ring. Con A also agglutinates erythrocytes, although not as powerfully as PHA. It preferentially stimulates the suppressor T cell population (Roitt et al,1985).

Pokeweed Mitogen (PWM) is extracted from several parts of the pokeweed (Phytolacca americana), and differs from Con A and PHA in that it has little erythroagglutinating activity. It stimulates both T and B cells (Greaves & Janossy, 1972; Douglas,

1972) and, in view of the fact that only one B cell in ten thousand responds to specific antigen, PWM has become important in in vitro studies of B cells (Chiorazzi et al, 1982).

Other compounds, apart from the lectins, have mitogenic properties. Protein A has been claimed to be a highly efficient mitogen for human peripheral B lymphocytes with no detectable activity for T lymphocytes (Forsgren et al, 1976). However, Sakane & Green (1978) found that it was a mitogen for both B and T lymphocytes.

Dextran sulphate has been shown to activate lymphocytes (Ruhl et al, 1974), and is a B-cell polyclonal activator (Gronowicz et al, 1974). Tuberculin PPD at low concentrations acts as a specific antigen, stimulating only lymphocytes of sensitized animals. However when used in high concentration it acts as a polyclonal B-cell activator (Gronowicz et al, 1974).

Lipopolysaccharides (LPS) from Gram negative bacteria, stimulate T and B cells in humans and only B cells in mouse. They induce differentiation of antibody-producing plasma cells. The lipid, rather than the polysaccharide moiety of the LPS, has been demonstrated to be responsible for mitogen activity (Andersson et al, 1973; Peavy et al, 1973; Rosenstreich et al, 1973).

It has been postulated that lymphocyte proliferation can be induced by the Fc-fragment of immunoglobulin (Berman et al, 1979) and by a wide range of other substances including periodate ions (Novagrodsky & Katchalski 1973), zinc ions (Ruhl et al, 1971;

Berger and Skinner, 1974), mercury ions (Ruhl et al; 1971), proteolytic enzymes and extract of polymorphonuclear lymphocytes (Mazzei et al, 1966).

Although plant lectin-induced mitogenesis is a rather artificial event, there is no doubt that it has enormous practical usefulness. Polyclonally-activated lymphocyte subpopulations express differentiated functions, and polyclonal activation only amplifies the changes associated with proliferation and permits their detection.

4.2. General Mechanism of Activation

There are many changes in metabolic activities of lymphocytes between initial stimulation by mitogens and the start of DNA synthesis. Biochemical studies have shown that there are alterations in the activities of almost every metabolic pathway, some of which occur more rapidly than others. Metabolic changes result in the enlargement of the cell (in particular in the cytoplasm), in the conversion of much of the nuclear heterochromatin to euchromatin and in the appearance of prominent nuclei (Ling, 1978).

4.3. Receptor - Membrane Interaction

The response of lymphocytes is initiated by binding of mitogen to receptors on the surface of the responsive cell. The response does not require passage of the stimulatory agent into the cytoplasm but depends upon the transmembrane transmission of

information and possibly on the generation of a "second messenger". Responses at the plasma membrane itself may be related to mitogen-receptor interaction (Wedner and Parker, 1976).

Two basic parameters determine the ability of an individual cell to respond to an external stimulus: the concentration of the external ligand in the extracellular fluid and the concentration of high affinity receptors for that ligand on the cell surface. It is now well established that cells have the ability to regulate the density of their plasma membrane receptors and thereby to alter their sensitivity to particular ligands. "Down regulation" describes the cells' response to high doses of ligand by reducing the density of its receptors; "Up regulation" denotes the acquistion of new receptor specificities as a consequence of cell activation e.g. Insulin receptors, which are normally absent, or present in very low concentrations on T cells, can be accquired (Helderman et al, 1977).

Mitogenicity depends upon the multivalency of the inducing ligand, and its ability to cross link the membrane components (Prujanski et al, 1978). This is the generally accepted view, although a number of monovalent agents having mitogenic properties have been reported. Fraser et al 1976 prepared a monovalent derivative of Con A which retained its mitogenic activity. Sela et al (1976) suggested that mitogens which are monovalent can become effectively multivalent when concentrated

and presented to the responding lymphocyte on the surface of the macrophage.

4.4. Proliferation parameters

4.4.1. DNA synthesis

DNA synthesis is not a continuous process even in cells which are undergoing rapid division (Wedner and Parker, 1976). It is, confined to one period of the cell cycle, the S-phase. In a normal cell cycle, mitosis is followed by a growth phase, G1, when no DNA is synthesised. The next stage is the S-phase, then the G2 phase where DNA synthesis has been completed, and the cell again prepares for mitosis. As DNA synthesising cells are rare in many populations of lymphocytes, the initiation of DNA synthesis has proved a sensitive low background assay that has become a standard method of determining lymphocyte transformation. The incorporation and transport of thymidine into DNA of lymphocytes stimulated by PHA is virtually confined to those cells which have DNA contents between the diploid (GI) and tetraploid (G2) values. Peters and Hausen (1971) showed that thymidine enters the cell by carrier-mediated diffusion, with the response occurring after a 24 hour lag. Feulgen-stained smears, and isotope incorporation determined by autoradiography showed that DNA synthesis was continuous throughout S phase only.

The most frequently used radioactive precursors for the study of DNA synthesis induced by mitogen stimulation are those

containing ^{3}H and ^{14}C isotopes of thymidine. The main differences between these are that ^{3}H-thymidine is available at a much high specific activity and is significantly cheaper. Other nucleotides such as deoxycytidine, deoxyuridine and iodo-deoxyuridine have been used (Ling, 1974). The increase in DNA synthesis resulting from the proliferation is assessed by measuring the incorporation of radio-labelled nucleotide into acid-precipitable material.

The rate of thymidine incorporation depends upon the concentration of thymidine added. Saturation occurs at a thymidine concentration of about 20ug/ml(Sample & Chretien, 1971). Most workers use lower concentrations (0.1ug/ml) because high thymidine levels inhibit DNA synthesis (Hartog et al, 1967). However, short pulses (4h) of high concentrations of thymidine produce reliable results for the synthesis of DNA. DNA synthesis after addition of PHA begins after a lag of 24 h and is usually found to be maximal after 72 h, dying away at later times (Hardy & Ling, 1973).

An enzyme immunoassay has been used by Porstmann et al (1985) to measure lymphocyte proliferation. They used an analogue of thymidine, 5-bromo-2-deoxyuridine (BUdR) which is incorporated into DNA and measured by using an anti-BUdR antibody. The results obtained were as sensitive as [^{3}H]-thymidine incorporation.

Many factors can affect the onset, extent and duration of DNA synthesis. These include the nature, concentration and metabolism of the stimulant used, the source and prior history of the responding cells and the culture conditions.

4.4.2. RNA synthesis

Lymphocytes have a large stable pool of inactive mRNA (Wettenhall et al, 1976). Liver and muscle cells also have a similar pool of mRNA (Wool & Cavicchi, 1966). This stored mRNA has very low template activity which increases rapidly following the addition of mitogen, which is (Wettenhall et al, 1976) responsible for the adenylation of mRNA (Hauser et al, 1976) promoting transport of RNA from the nucleus to the cytoplasm (Mitchell et al, 1976). Both these increases reflect an overall increase in the rate at which mRNA is processed.

Free ribosomes, present in large numbers in unstimulated lymphocytes, rapidly form clusters and polysomes during transformation (Cooper, 1977; Harms - Ringdahl & Cooper, 1978). This ribosome aggregation may be related to the phosphorylation of a specific ribosomal protein that occurs within 20 minutes of Con A addition (Wettenhall et al, 1979). A similar response is observed as chick embryo fibroblasts enter G1 in response to the addition of insulin or growth factors (Haselbacher et al, 1979).

In stimulated lymphocytes the pattern and extent of methylation of tRNA is significantly altered. Methylation of tRNA may be necessary as a preliminary step in the acceptance of amino acids by transfer RNA, in the binding of aminoacyl tRNA to ribosomes and in the codon response.

Most living lymphocytes are engaged in RNA synthesis. If

highly sensitive autoradiographic methods are used, a high proportion of cells can be shown to incorporate the pyrimidine nucleosides, tritiated uridine, and tritiated cytidine. Torelli et al (1964) showed that different morphological classes of human blood lymphocytes may have different rates of incorporation.

When lymphocytes are stimulated with PHA, the rate at which they incorporate ^{3}H-uridine, and ^{3}H-cytidine into RNA is greatly increased. The nucleosides enter by carrier mediated facilitated diffusion. This begins soon after stimulation and becomes very prominent well before the initiation of DNA synthesis (Epstein & Stohlman, 1964). Similar increases have been reported with many other mitogens and seem to be a universal feature of activation. There have been no reports of DNA synthesis in lymphocytes not preceded by a stimulation of incorporation of pyrimidine nucleosides into RNA.

Most workers observed an increase in the rate of ^{3}H-uridine incorporation into RNA within one hour of the addition of PHA. In some cases the increase has been small, and in others as much as four fold (Cooper & Rubin, 1965; Kay & Cooper, 1965). The explanation is probably that the optimum concentration of PHA required for DNA synthesis may be lower than that required to initiate RNA synthesis.

The incorporation of other precursors into RNA has been studied less intensively. PHA increases the rate of ^{32}P-phosphate incorporation into RNA, although the stimulation is smaller than

that seen with ^{3}H-uridine (Ebaugh, 1962). The stimulation of the incorporation of purine nucleosides, adenosine and guanosine into RNA is of the same order as that of ^{3}H-uridine but unstimulated lymphocytes incorporate the adenine into RNA much more readily than the adenosine, and the incorporation of adenine was stimulated much less by PHA. When cycloheximide inhibits RNA synthesis, uridine is still taken up, indicating that the uptake does not depend upon RNA or protein synthesis. Therefore, increases in uridine labelling at various times after stimulation should take into account uridine transport and phosphorylation. The availability of intracellular nucleosides or nucleotides may limit the RNA synthesis.

4.4.3. Protein synthesis

A sharp increase in the transport of amino acids across the plasma membrane is a universal feature of the response of mammalian cells to growth stimuli (Cunningham and Pardee et al, 1978; Dubrow et al, 1978). The carrier systems that transport amino acids are termed the A system and the L systems. The former, but not the latter, utilises the plasma membrane Na(+) gradient to drive uptake against a concentration gradient (Van den Berg and Betel, 1972). The omission of amino acids from the medium results in the inhibition of mitogenesis (Van den Berg, 1972) suggesting an important requirement for exogenous amino acids at one or more stages of transformation.

The rate of turnover of protein may also be affected. Tanaka

& Ichihara (1976) found a three fold increase in the half lives of several proteins that are rapidly turned over in stimulated L - cells. The increase correlated with an increase in the activity of a lysosomal enzyme involved in protein degradation. This possibility may apply to lymphocytes and is consistent with increased lysosomal activity actually observed in PHA stimulated lymphocytes (Alison & Mallucci, 1964).

The addition of mitogens to lymphocytes is followed rapidly by the stimulation of radioactive amino acid incorporation into protein. The reported time courses of amino acid conversion into protein varies with different authors. Incorporation of ^{14}C-leucine into acid-precipitable material has been observed within five minutes of addition of Con A to rat thymocytes (Yasmeen et al, 1977). Pogo et al (1966) found the incorporation of radio-labeled alanine after 15 minutes exposure to PHA of human peripheral blood lymphocytes. Hausen et al (1969) studied the uptake of radiolabelled amino acids into intracellular protein of PHA-stimulated peripheral blood lymphocytes, and observed an increase in incorporation of radiolabel within two hours of PHA addition. Levy et al (1973) could only detect the incorporation of other radiolabelled amino acids into protein after two to three hours. However, the majority of workers observed increases after two hours continuing after forty-eight hours. Facilitation of ribosome processing has been observed as early as one hour after PHA addition to lymphocytes. This movement of protein and

RNA in and out of the nucleus in stimulated lymphocytes suggests that the nuclear membrane may have an important regulatory function. The rate of incorporation of amino acids seems a reasonably accurate index of the rate of protein synthesis (Schechter et al, 1973) at least when the amino acids leucine or phenylalanine are used (Kay et al, 1971). ^{3}H-leucine incorporation in particular has been used (Levy & Kaplan, 1974) and recommended as a more sensitive indicator of lymphocyte abnormality than the more common ^{3}H - thymidine uptake assay (Behan, 1979).

The variation in results obtained from different laboratories possibly reflects the use of different culture media, PHA concentration, labelling protocols and labelled amino acid chosen.

4.4.4. Carbohydrate Metabolism

Only glucose or closely related molecules can supply the energy needed to support long term cell multiplication in culture (Fodge & Rubin, 1973). Moreover, glucose is a source of many intermediates needed for nucleic acid, carbohydrate and protein synthesis as well as providing the reducing power needed in lipid synthesis. Because all of these processes are required for multiplication, glucose might be expected to play a key role in co-ordinating these activities.

Apart from an overall stimulatory effect of cell metabolism,

glucose could have a more selective function in providing energy or substrate required in the synthesis of a critical regulatory molecule e.g. in the thermocytes, glucose has been shown to be required for maximum incorporation of radiolabelled amino acids into proteins in the nucleus (Giddings & Young, 1974).

D-glucose enters the lymphocyte by carrier-mediated facilitated transport. In their studies of the effect of PHA on the transport of a non-metabolisable analogue of glucose, 3-O MG, Peters & Hausen (1971) found an increase in the rate in influx after two minutes, and a five fold increase after thirty minutes suggested that PHA acts by making more carrier molecules available to the glucose.

Most mammalian tissues do not produce large amounts of lactate when they are incubated under normal aerobic conditions. Otto Warburg (1926) was the first to report high rates of lactate production by rapidly proliferating cells. He observed that white blood cells oxidising glucose under aerobic conditions, showed high rates of lactate production. It has been suggested that the glycolytic capacity of growing cells may be regulated by the co-ordinated control of the expression of glycolytic enzymes. Lactate production by lymphocytes increases to four times the initial rate after 24 hours following PHA addition (Packman, 1968). The effect of PHA increases with time, and early changes in carbohydrate metabolism include the accumulation of lactate, pyruvate,

does occur and as much as 85% of ATP synthesized may result from oxidative phosphorylation. (Roos & Loos, 1973). From 30 to 120 min there were increases in parameters of carbohydrate metabolism (Peters and Hausen, 1971). Early metabolic changes can be measured by assays of glucose consumption (De Cock et al,1980) or lactate production (Cordiali - Fei et al, 1980; Polgar et al, 1968; Roos & Loos,1970; Parkes & Howells, 1975). De Cock et al (1980) found that glucose consumption correlated well with ^{3}H-thymidine incorporation into DNA, and morphological studies of transformed cells. Their test was highly reproducible registering the whole event of transformation, unlike ^{3}H-thymidine incorporation or morphological studies. The rate of glucose uptake doubles within 15-30 minutes of PHA addition and increases further with time (Peters & Hausen, 1971).

Lactate release can also be monitored Cordiali - Fei et al (1980) found significant levels of lactate released after three hours of stimulation with PHA of peripheral blood lymphocytes. The results were reproducible and correlated well with ^{3}H - thymidine incorporation into DNA. Lactate release continues with PHA stimulation.

4.4.5. Others Parameters

The increase of Ca^{2+} influx appears to be one of the earliest events which takes place following contact with stimulating agents (Kennes et al, 1981) and uptake of radiolabelled calcium was found after 30 min. Kennes et al, (1981) and Freedman et al (1979) both

used the assay and found it was variable but correlated quite well with ^{3}H-thymidine incorporation.

The insulin receptor can also be used as a marker for activation. It is not normally present on T and B cells but appears on the membrane as a consequence of activation. The insulin receptors appeared after three days of culture with the stimulant, and are easily monitored with ^{125}I- insulin, (Helderman et al, 1978). The insulin receptor has been shown on lymphocytes from man, mouse and rat upon activation, and is therefore a general marker of cellular activation.

Section 5. Human Monoclonal Antibody Production

Section 5 Human monoclonal Antibody Production

5.1. Introduction

The theory of monoclonal antibody production is based on the clonal selection hypothesis of Macfarlane Burnet (Burnet, 1959). Each mammalian B cell has the potential to make a monospecific antibody. The constant region of the antibody chain may alter but the variable region retains its singular specificty.

The first report of hybridoma production was in 1970 when Sincovirs et al, (1970) fused virus specific lymphocytes with tumour cells. However, the full potential of the monoclonal technology was developed in a now famous paper by Kohler and Milstein in 1975.

5.2. Monoclonal Antibodies by Cell Hybridisation

5.2.1. Theory

The experimental problem encountered in the production of monospecific antibody relates to the fact that antibody secreting plasma cells are terminally differentiated lymphocytes with a finite life span, and cannot be grown in culture. However, tumours of such cells can be found in animals, and can be readily induced in mice with the aid of mineral oils (Kohler and Milstein, 1975, 1976; Campbell, 1984). These tumour cells secrete antibody of a single specificity yet can be grown indefinitely in culture. If such tumour cells can be fused with a lymphocyte making antibody

of a required specificity some hybrids will have the eternal growth of one parent, and specific antibody production of the other (Kohler & Milstein, 1975, 1976). There are two key refinements of the technique; firstly, the selection of a tumour parent line which does not itself secrete antibody, so the hybrid antibody is necessarily that of the required specificity and secondly selection of a cell line that is vunerable to the cell culture conditions so that it cannot survive unless it has participated in the fusion. The most frequently used way of achieving the latter condition is to use a parent tumour line which lacks either the enzyme thymidine kinase (TK) or hypoxanthine phosphoribosyl transferase (HPRT). These enzymes are from the salvage pathway of nucleic acid metabolism and are essential for cells growing in the presence of aminopterin which blocks the major pathway of nucleotide synthesis. The cells after fusion are cultured in media containing Hypoxanthine, Aminopterin, and Thymidine (HAT) (Littlefield, 1964) so that the parent tumour cells die; hybrid cells possess the salvage enzymes of the non-tumour parent and survive. Methods Figure 2.1 shows a schematic representation of the fusion procedure. The hybrid cells are grown in culture plates and assayed for production of the required antibody. The suitable clones are then selected for expansion and subcloning so that eventually the required antibody can be produced in large amounts.

5.2.2. Fusion Partner

Both mouse and rat systems are widely used and the choice between the two is fairly open. The human hybridomas are harder to make than are the rodent ones and are only produced when the other systems cannot be utilised.

The mouse system was historically the first to be developed as a method of production of monoclonal antibodies of predefined specificity (Kohler & Milstein, 1975) and the majority of hybridomas to date are of mouse origin. Table 1 lists some of the mouse cell lines currently available as fusion partners. All the cell lines originated in Balb/c mice. The most obvious choice of mouse species for immunisation would be Balb/c as this is compatible with the myeloma cell lines, and the hybridomas produced can be grown in ascites fluid.

The rat system has been reported to have definite advantages. The rate of reversion of cell lines to non-secreting forms is lower than in presently available mouse cell lines, and this is obviously a valuable characteristic if it is retained in the progeny. An additional advantage is that 90% of the growing hybrids express spleen immunoglobulins compared with only 60% in the mouse system (Clark et al, 1983). Rats are larger and yield approximately ten times more ascitic fluid in the production stages (Galfre et al; 1979).

Additional advantages of the rat system may lie in the high frequency of antibodies able to fix human complement. If

therapeutic applications are envisaged then this property may be significant.The rat system was originally developed by Bazin (Bazin et al, 1972,1973;). Rat myelomas are comparatively rare.

Human hybridomas present more difficulties and their use is only really justified if the final application of the monoclonal antibody precludes the use of mouse or rat (Edwards & O'Hare, 1984). There are few non-secreting human myeloma, or lymphoblastoid cell line presently available and this leads to mixed and diluted antibody production. Only a few papers have reported the production of human-human hybridomas(Croce et al, 1980; Olsson and Kaplan, 1980; Shoenfeld et al, 1982; Sikora et al, 1982) and relatively few deal with technical details (Olsson et al, 1983). Shoenfeld et al (1982) stimulated mononuclear cells from the spleen or peripheral blood with PWM, favouring autoantibody producing B-cells. Their human-human fusions gave low frequency of hybridomas but the frequency of autoantibody producing hybrids was comparable to that of mouse-mouse hybrids. The results of Olsson et al (1983) confirmed the low hybridoma frequency but this was found to be comparable with that from fusions of mouse peripheral blood lymphocytes with a corresponding mouse myeloma cell line. They also studied the use of PWM over a period of days before fusion and observed that, if the peripheral blood lymphocytes were stimulated for 5-7 days, the number of hybrids was significantly increased. The reasons for this probably stems from the fact that when PEG is used to fuse the cells it

only mediates the fusion of the cell membranes, whereas the fusion of the karyons occurs spontaneously when the two nuclei synchronously enter mitosis. However, human peripheral blood lymphocytes are not normally in active cell cycle and the fusion of such cells with a human myeloma/lymphoma cell line results in very few successful hybrids as very few are actually in mitosis. The number of hybrids was increased with PWM because more cells are actively dividing when fused. The number of Ig-secreting clones tended to be higher with PWM-PBL but not significantly. While hybrids of the appropriate specificity are sometimes obtained after the initial fusion, subcloning is necessary to maintain secretion, and hybrids present many technical difficulties.

Clearly,humans are not generally immunised for monoclonal antibody production and human hybridomas are of interest more for the study of autoimmune or malignant disease.

Many early fusion experiments were often carried out with mouse cell lines. These interspecies fusions were first reported in 1973 (Schwaber and Cohen, 1973) when the mouse myeloma cell line TEPC-15 was fused with human PBL and the heteromyeloma secreted both human and mouse immunoglobulin. Similar fusions have been performed since (Schwaber and Rosen, 1978; Croce et al, 1979; Astaldi et al, 1980; Nowinski et al, 1980; Sclom,Wunderlich and Teramoto, 1980; Lane et al, 1982; Bron et al, 1984; Foung et al, 1984). Many of these were generated from spleen cells, lymph nodes

and PBL.

Mixed human-human and human-mouse hybrids tend to lose human chromosomes preferentially although human chromosome 14 (carries the heavy chain locus) and 22 (which carries the lamba chain locus) are frequently retained (Croce et al, 1980). Loss of secretion has occured even when the relevant chromosomes have been retained (Raison et al, 1982). The loss of antibody production was found not to be due to the overgrowth of the secreting clones by the non-secreting clones, but rather by a loss or inactivation of antibody-producing chromosomes (Westerwould et al, 1983). The greatest loss in secretion results in the first three weeks after fusion and then stabilises. This unstability in mouse-human hybrids has made them unpopular as a method of human monoclonal antibody production. Despite all these findings, Cote et al (1983) have shown that if early and repeated subcloning is carried out, the mouse - human system can yield hybridomas with stability comparable to that of human - human fusions. The use of the mouse system enables one to employ non-secreting cell lines, not yet possible with human hybridomas. To date only a limited number of human monoclonal antibodies have been reported (Lagace et al, 1985). There are four main cell lines available for use in human - human fusions (Edwards & O'Hare 1984) SKO-007, GM1500, LICR-LON-HYM2, UC729.

5.2.3. The Selection of Cell Lines

There are several factors to consider when choosing a cell line. As mentioned already it is preferable to choose a myeloma cell line that does not secrete antibody. If a myeloma makes only light chains then the amount of irrelevant antibody is limited and at least 25% will be the immunoglobulin of the desired specificity. However, if both heavy and light chains are secreted then specific antibody is more diluted. Generally ,most fusions are mouse-mouse and the most common myelomas used are P3X63.Ag8-653 and NS1 Ag4-1, of which NS1 produces intracellular light chains. Since these can combine with heavy chains produced in a hybridoma and then secreted, the preferred myeloma is Ag8. Another line that is often used is SP2. However, SP2 is not a true myeloma but a non-producing hybridoma and as such, in theory, less stable. Table 1 gives a summary of the cell lines available for fusion and their properties.

Table 1. A Summary of the Plasmacytoma Cell Lines used for Hybridisation

Cell line	Myeloma parent	Drug resistence	Immunoglobulin Chain H	L
P3X63-Ag8 (P3)	MOPC 21	8-Ag	yl	k
P3/NS-1/1-Ag4-1 (NS-1)	P3	8-Ag	-	k
Sp2/0-Ag14 (Sp2)	P3+spleen	8-Ag	-	-
FO	Sp2 = Sp2	8-Ag	-	-
P3x63-Ag8.653	P3	8-Ag	-	-
MOPC-315LK	MOPC 315	none	a	L2
4TOO.1	MPOC 11	6-TG + oubain	y2B	k
P326BU4	MOPC 21	Brdu +	yl	k
PBOO.1	p326BU4	BrdU + oubain	yl	k
C1.3	X5563	none	y2A	k
104E	MOPC 104E	none	u	L
45.6TG1.7	MOPC 11	6-TG	y2B	k
4TO.2	MOPC 11	6-TG + oubain	Y2B	k
P1BU1	Adej.PC5	BrdU	Y2A	k
P1BU1-Ou	P1BU1	BrdU + oubain	Y2A	k
P1BU2	Adj.PC5	BrdU	-	k

S194/5.xx0.BU.5 (S194)	S194	BrdU	-	-
TEPC-15	TEPC-15	none	a	k
210.RC43.Ag1	Rat	8-Ag	-	k
Y3-Ag1.2.3	Rat	8-Ag	-	k
UM.1-6TGr	UM-1	6-TG	u	k
Um-21-5	UM-21	citrullinemic	-	k
GM 1500 6TG-A12	GM 1500	6-TG	Y2	k
U-266AR1	U-226	8-Ag	E	L

From J. Imm Methods 53 (1982) pp.261-291

5.2.4. The Fusion Procedure

The basic theory behind monoclonal antibodies revolves around two important discoveries. The first was to select an agent that would cause the fusion of two cells (the myeloma and the spleen cell), and the second was to develop a selection method to separate those cells that had fused to give a hybrid from unfused spleen and myeloma cells.

In their original paper Kohler & Milstein 1975(Nature 256:495) used Sendai virus as the fusion agent. This virus has the ability to cause the two membranes to fuse. It was found that polyethylene glycol would do the same thing but was much easier to handle and so has been the fusion agent of choice ever since (Pontecorvo, 1976; Lane, 1985; Roitt et al,1985).

The most common cells used in the fusion are spleen cells. However, peripheral blood lymphocytes can also be used. The latter are separated on ficol-hypaque gradients to remove the red blood cell contamination. The spleen cells naturally die in culture after 1-2 weeks;the myloma cells die in HAT, but the fused cells survive having the immortality of the myeloma and the metabolic by-pass of the spleen cells.After the inital 1-2 weeks in HAT the cells are then transferred to HT medium, which lacks the powerful toxin Aminopterin, for 1 week and then finally to culture medium.

5.2.5. Screening

When screening, it is essential to identify desirable hybrids

quickly, as the labour of maintaining and characterising them is greater than that needed to perform another fusion. The screening assay must be sensitive to about 1ug of antibody /ml.

Antibodies are commonly detected by ELISA or RIA based on immobilised antigen (Williams et al, 1967; Goding, 1980). However, the antibody should be tested in the way that is is to be used. e.g. immune precipitation of antigen has been used for screening after a preliminary rapid test for abundant antibody binding to protein A of Staphylococcus aureus (Brown et al, 1980). Staining of tissue sections, or cells by immunofluorescence (Goding, 1980) or immunocytochemical methods (Schlom et al, 1980) are suitable screening methods for antibodies that mark particular cells or special structures such as intermediate filaments.

5.2.6. Plating and Cloning

Hybridomas are commonly lost between detection of postitive clones and cloning. The fused cells carry a tetraploid number of chromosomes making them unstable and therefore have to expend large amounts of energy in order to replicate. Cells which lose chromosomes can divide more quickly, but the chromosome lost may be the one required for immunoglobulin secretion. Hence the non-secreting clones will overgrow the desired hybridomas. Cells secreting immunoglobulin also use substantial amounts of energy for protein synthesis and therefore have less energy for cell

division. Early cloning is essential for maintaining immunoglobulin secretion.

Cloning a single hybrid under adverse conditions is time consuming. If one is screening for an antibody that discriminates between 2 antigens, a specific clone may be missed if it is mixed with a clone that produces non-specific antibodies. Hence fusion protocols should be designed to generate clonal hybrids from the outset, hybridomas are enhanced in both cell growth and antibody production by various specific growth factors (Astaldi et al, 1981; Pintus et al, 1983; Westerwould et al, 1983). These observations have led to the extensive use of feeder layers (macrophages or thermocytes, non-dividing cells that provide a suitable environment, Goding et al, (1980)) during hybrid production (Goldsby et al, 1978; Kennett et al, 1978; Brodin et al, 1983; Teng et al, 1983; Strike et al, 1984). The feeder cells presumably condition the medium and optimise it for hybridoma cell growth and survival. Long et al (1986) found that irradiated human diploid cells were highly effective in the establishment and propagation of new hybridomas and in maintaining viable hybridomas during limiting dilution. Human-human fusions always have feeder cells present (preferably human) as this gives the hybrids the most possible help. Only 10% of wells should contain hybrids after limiting dilution (Fazekas de St. Groth et al, 1980). An alternative is to plate the hybrid cells in agar so that they grow as clones (Sharon et al, 1979; Edwards et al,

1980). Individual positive clones are picked from wells and retested after isolation (Edwards, 1980). Hybrids should be recloned by limiting dilution, rather than in agar which gives a lower cloning efficiency and may select for unwanted cells that are more vigorous than the desired hybrids.

Pokeweed Mitogen Stimulation of B Lymphocytes

Mitogenic stimulation of B-cells results in a greater chance of obtaining antibody-secreting hybrids after fusion or transformation. A number of reports (Astaldi et al, 1980; Olsson et al, 1983) suggest stimulation with PWM. This mitogen is known to induce human peripheral blood lymphocytes to enter S phase and also to induce B lymphocytes to differentiate into plasma cells (Janossy et al, 1975; Ginsburg et al,1978; Tosato & Blaese, 1985). While differentiation of plasma cells usually reaches a maximum after 6-7 days, stimulation with PWM reduces the period to only 4 days (Yen et al, 1981). PWM-induced polyclonal B-cell activation requires the participation of helper T-cells (Keightly et al,1976; Fauci et al,1976; Hirano et al, 1977; Saxon et al, 1977) or their secreted products (Insel et al,1977) while suppressor T-cells can modulate generation of immunoglobulin synthesising cells (Moretta et al,1977; Lipsky et al, 1978). This mitogenic stimulation of B-cells results in a greater chance of obtaining antibody secreting hybrids after fusion or transformation.

5.2. The Production of Human Monoclonal Antibodies by EBV Transformation

Epstein-Barr is a ubiquitous virus that infects the majority of human adult normal individuals worldwide (Tosato and Blaese,1985). Primary infection with the Herpes virus is generally asymptomatic, particularly in childhood, but may result in acute infectious mononucleosis in adults (Henle and Henle,1979). One of the main characteristics of the virus is that it induces normal human B lymphocytes to proliferate and secrete antibody,and to become activated into long term cell lines that can be propagated in vitro for years (Sugden,1982; Nilsson and Klein,1982). That is, EB virus is a polyclonal B cell activator. The induction of the indefinite cellular proliferation in vitro by EBV is termed "transformation".

EBV transformation offers an alternative method of obtaining monoclonal human B-cells that secrete human monoclonal antibodies of defined specificity. The great advantage of this method is that a very high percentage of antibody producing cells may be immortalised and no chemical selection is required. This technique has been used to obtain human monoclonal antibodies to tetanus toxin (Kozbor et al,1982), red blood cells, rheumatoid factors, and anti-nuclear antibodies in SLE.

Infection of lymphocytes with the virus represents a necessary step to achieve B-cell activation and immunoglobulin production. Culture supernatant of a Burkitt lymphoma cell line,

marmaset B95-B, is the most commonly used source of the virus (Tosato and Blaese,1985). The continuous presence of the virus is not necessary for transformation and a 1-9 hour exposure is usually sufficient (Yarchoan et al,1983). In contrast to other B-cell polyclonal activators, EBV does not require cooperation from other cells e.g. T-cell or macrophages (Kirchner et al,1979). During the first 8 days in culture the B-cell response to EBV in vitro is not significantly different from the response to other B-cell activators such as PWM. All the major classes and subclasses of immunoglobulin (Andersson et al,1981) are produced and production can increase exponentially over a 14 day culture period. However, in most cases B-cell response is inhibited after 8 days because most individuals produce cytotoxic T-cells which attack and destroy the virus infected B-cells (Tosato et al, 1982). Hence for successful transformation, removal of this cytotoxic effect is necessary. This can be achieved in a number of different ways. T lymphocytes can be removed by E-rosetting with sheep red blood cells (Roitt et al, 1985), by killing them with drugs, e.g. cyclosporin A (Tosato et al, 1984a) or, better still, the B-cells may be positivly selected. Positive selection is readily performed with soluble antigens which are attached to SRBC and then separated on percoll gradients.The relevant lymphocytes are then located in the pellet (Kozbar & Roder,1981).

There are several disadvantages in this technique. One is that B-cell immortalisation by EBV is not synonymous with

immunoglobulin production. In fact, most (approximately 95%) of B-cells infected with EBV secrete immunoglobulin but only 60 % become immortalised (Tosato et al, 1983). Immunoglobulin production by EBV-activated cells that do not give rise to long-term antibody production could be due to a phenomenon of abortive transformation (Tosato & Blaese,1981). The other disadvantage is the very low level of immunoglobulin secretion of EBV transformed cells which is rarely above 1µg/ml of culture supernatant (most commonly 10-100ng/ml). Both problems may be overcome by early cloning of the transformed cell or much better by fusion with a myloma cell line (Kozbor et al, 1982 used KR-4 cell line).

5.3. Monoclonal Antibodies and Autoimmune Disease

Current concepts of autoimmunity emphasize the role of antibodies in the development and maintenance of most autoimmune disorders (Allison, 1977) including SLE, RA, MG, autoimmune thyroiditis, autoimmune haemolytic anemia and several experimental and drug induced autoimmune diseases. With the exception of a few monoclonal rheumatoid factors, the majority of autoantibodies are polyclonal.

Consequently, the detailed investigations of the structure, specificity and pathogenic role of these antibodies has been very difficult. Hybridoma technology presents the opportunity of studying autoantibodies in more detail.

5.3.1. Antigen Specificity of Autoantibodies

A precise characterisation of antigens involved in an autoimmune syndrome may help in understanding its etiology and pathogenesis with eventual diagnostic and prognostic applications. Autoantigens have been identified in a number of autoimmune diseases, including the acetylcholine receptor in MG, (Lindstrom,1979), IgG in RA (Zvaifler, 1977), and nuclear antigens in SLE (Stoller,1973). It should be borne in mind however, that the ability to find autoantibody does not necessarily identify the true initiating autoantigen (Moller et al, 1981). In SLE, the putative autoantigen is DNA which possesses many antigenic determinants. Autoantibodies that bind to DNA could be directed against any number of determinants.

The presence of autoantibody-producing B-cells in healthy mammals is supported by the existence of animal models. Also administration of polyclonal B-cell activators to normal animals gives rise to detectable antibodies to DNA, and poly A (Fourie et al, 1974; Fischbach et al, 1978).

There are many problems concerning the role of autoantibodies in mediating disease. A given autoantibody could be an essential part of a pathological mechanism which leads to tissue damage and organ dysfunction, or it may appear as a product of the disease.

Monoclonal antibodies, when isolated and purified in sufficient quantities, may contribute to the eludication of a

pathogenic mechanism. The production of several monoclonal antibodies that possess the same antigenic specificity, but differ with respect to antibody class, and subclass, antibody valence, degree of polymerisation and complement-fixing properties may help to determine the relative importance of each of these parameters in mediating autoimmune disease in experimental animals. Also the special characteristics of the interactions between autoantibodies and their antigens is important in the expression of pathological manifestations, particularly in autimmune diseases which are classified as immune complex diseases.

CHAPTER 2 MATERIALS AND METHODS

2. Material and Methods

2.1 Materials

2.2 Methods

2 Materials and Methods

2.1 Materials

2.1.1. Radiochemicals

[Thy-methyl-3H] (specific activity 25 Ci/mmol); [Leu-4,5-3H] (specific activity 64 Ci/mmol); [Urid-5,6-^{3}H] (specific activity 40 Ci/mmol); were purchased from the Radiochemical Centre, Amersham, Bucks; U.K.

2.1.2. Enzymes

Deoxyribonuclease I (bovine pancreas), trypsin (bovine pancreas), horseradish peroxidase and alkaline phosphatase-conjugated antisera were purchased from Sigma Chemical Company, Poole, Dorset, U. K.

2.1.3. Mitogens

Concanavalin A, and Phytohaemagglutinin were obtained from Pharmacia, Uppsala, Sweden. Pokeweed Mitogen was obtained from Sigma Chemical Company, Poole, Dorset, U.K.

2.1.4. Cell Lines

P3.X63-Ag8-653 a non-immunoglobulin secretor mouse myeloma B-cell line was obtained from Flow Laboratories, Irvine, Scotland

2.1.5. General Reagents

The following items were obtained from Flow Laboratories: fetal calf serum; donor horse serum; L-glutamine; penicillin-streptomycin; RPMI 1640; leucine-free minimal essential medium; phosphate-buffered saline; sodium pyruvate; sodium bicarbonate;

Lymphocyte Separation Medium; HAT; HT; and 10-spot multi test slide.

Dulbecco's Minimum Essential Medium (DMEM); high binding microtitre plates, and all sterile plastic ware were purchased from Gibco-Nunc, Paisley, Scotland.

The following items were purchased from BDH, Poole, Dorset, U.K.: Trypan blue; acetic acid; perchloric acid; trichloroacetic acid; eosin; Delafeld's haematoxylin; microscope slides; coverslips; n-propyl gallate; polyethylene glycol. Di-nitrophenyl phosphate (DNPP) and all other chemicals were Analar grade.

The assay kits, for glucose and lactate determination and uranyl acetate were obtained from Boehringer-Mannheim, Lewes, E. Sussex, U.K.

2.1.6. Media

2.1.6.a) Lymphocyte Media

i) Lymphocyte Culture Medium

Components	Final Concentration
Fetal calf serum (heat inactivated)	10% (v/v)
L-Glutamine	2mM
Penicillin	100U/ml
Streptomycin	100µg/ml
RPMI 1640	85% (v/v)

ii) Leucine-free Lymphocyte Culture Medium

Components	Final Concentration
Fetal calf serum (heat inactivated)	10% (v/v)
L-Glutamine	2mM
Penicillin	100U/ml
Streptomycin	100µg/ml
MEM (Eagles)(Leucine free)	85% (v/v)

iii) Hepes-buffered Eagles Medium (H-Eagles)

Components	Final Concentration
L-Glutamine	2mM
Penicillin	100U/ml
Streptomycin	100µg/ml
Hepes	20mM
Sodium bicarbonate	0.85g/l
MEM	90 % (v/v)

PH adjusted to 7.4-7.6 with sterile 1N NaOH

2.1.6. (b) Neuronal Cell Culture Medium

i) Serum Supplemented Medium (SSM)

Components	Final Concentration
Fetal calf serum (heat inactivated)	10% (v/v)
Donor horse serum (heat inactivated)	10% (v/v)
L-Glutamine	2mM
Penicillin	50U/ml
Streptomycin	50µg/ml
Glucose	6.0g/l
DMEM	74% (v/v)

ii) Serum Free Medium (SFM)

Components	Final Concentration
Insulin	0.2U/ml
Human transferrin	5ug/ml
Progesterone	20nM
Putresceine	100µM
Sodium selenite	30nM
Hydrocortisone	0.5µM
Biotin	1µg/ml
L-Thyroxine	3µg/ml

This medium also contains all the components of SSM except the sera.

iii) Puck's Balanced Salt Solution

Components	Final Concentration(mM)
NaCl	137
KCl	5.4
Na_2HPO_4	0.169
KH_2PO_4	0.216
Glucose	33
Sucrose	44
Hepes	10

PH was adjusted to 7.3-7.4 with 1N NaOH

2.1.6 (c) Media for Monoclonal Antibody Production

i) Cloning Medium

Components	Final Concentration
Fetal calf serum	10% (v/v)
L-Glutamine	2mM
Penicillin	100U/ml
Streptomycin	100µg/ml
Sodium pyruvate	1mM
Mercaptoethanol	5x10-5M
RPMI 1640	83% (v/v)

ii) HT Supplemented Medium

This medium contains all the components of the cloning medium plus Hypoxanthine (0.1mM) and Thymidine (16µM).

iii) HAT Supplemented Medium

This is as the HT medium plus Aminopterin (0.4μM).

iv) Polyetheylene Glycol Solution (50% w/v)

Components	Final Concentration
Polyethelene glycol 4000 (MERCK)	10g
PBS	10ml
Dimethyl suphoxide (DMSO)	1ml

The PEG and PBS were measured into a glass container and then autoclaved , after which the DMSO was added.

2.1.7. Patients and Controls

Blood samples from patients with Motor Neurone Disease (MND) and Multiple Sclerosis (MS) were obtained from Dr. M. Campbell Consultant Neurologist at the Bristol Royal Infirmary. The patients were age and sex matched with healthy controls whenever possible.

2.2 METHODS

2.2.1 Lymphocyte Culture

2.2.1. (a) Lymphocyte Preparation

All the following procedures were carried out under aseptic conditions. Peripheral blood lymphocytes were isolated from whole blood by a modification of the method of Boyum (1968). Fresh venous blood (20ml) was collected into a sterile syringe, and expelled into a 30ml universal flask containing an anticoagulant, Heparin (20U/ml). The blood was diluted with an equal volume of phosphate buffered saline (PBS). This dilution with the PBS is carried out to control the degree of aggregation of the red blood cells so that they sediment easily with the minimum of lymphocyte entrapment. The diluted blood was carefully layered onto a gradient of lymphocyte separation medium in a 10ml polycarbonate centrifuge tube. After centrifugation at 400xg for 40 min at room temperature, the mononuclear cell layer was clearly visible at the interface as a white band. It was carefully removed with a 1ml Pasteur pipette, washed three times with PBS (20ml each wash), and finally resuspended at 2×10^6 /ml in lymphocyte culture medium (LCM). Cells used for the [^{3}H]-Leucine incorporation studies were resuspended in leucine-free lymphocyte culture medium.

2.2.2. Cell Viability

Lymphocyte viability was tested by the cells' ability to exclude trypan blue. Equal volumes of the cell suspension and 0.2% (w/v) trypan blue in saline were mixed, and the cells were counted using

a haemocytometer. The lymphocytes were counted in PBS rather than lymphocyte culture medium as the FCS in this medium could lead to false results because of the great affinity for trypan blue of proteins (Kruse et al, 1973). Counting was performed within 3 minutes after the addition of trypan blue. After this time viable cells also take up the dye.

2.2.3. Mitogen Stimulation

Samples (100µl, 2×10^5 cells) of the lymphocyte suspension were placed in the wells of U-bottomed microtitre tissue culture plates. An equal volume of Concanavalin A (Con A), Phytohaemagglutinin (PHA), Pokeweed Mitogen (PWM), or membrane fragments (see Section 2.2.8.) at the appropriate concentration was added. Control cultures received the lymphocyte culture medium only. The cultures were then incubated in a humidified atmosphere of 5% CO_2 in air at 37°C for 72 h. All the experiments were carried out in triplicate.

2.2.4. Lymphocyte Transformation Assays

2.2.4.1. Glucose Consumption Test

This was carried out according to De Cock et al (1980). At the appropriate time, samples (100µl) of the supernatant from both control and stimulated cultures were deproteinised with uranyl acetate (1ml) and centrifuged at 10,000xg for 5 min. The supernatants were then assayed for glucose within 24 h with a Glucose Test Combination Kit.

Test Principle

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{gluconate} + H_2O_2$$

$$H_2O_2 + \text{ABTS}^* \xrightarrow{\text{POD}} \text{Coloured Complex (610nm)} + H_2O_2$$

*ABTS = diammonium 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonate

GOD = Glucose Oxidase

POD = Peroxidase

Lymphocyte stimulation was expressed as the difference between glucose used by the control and stimulated cells.

2.2.4.2. Lactate Release

This procedure is very similar to that of the glucose assay. At the appropriate time, samples (200µl) of the supernatant from the stimulated and control cultures were deproteinised with ice cold perchloric acid (0.6M, 1ml), and centrifuged at 1000xg for 10 min at 4°C. The lactate content of the samples was measured by using the Lactate Test Combination Kit.

Test Principle

$$\text{L-Lactate} + \text{NAD}(+) \longrightarrow \text{Pyruvate} + \text{NADH} + \text{H}(+)$$

Stimulation was expressed as the difference in lactate content of the supernatants from the stimulated and control cells.

2.2.4.3 Incorporation of Radioisotopes

At the appropriate time, radiolabelled precursors (0.2μCi) were added to the cells in the lymphocyte culture medium (10μl) for a period of 18 h. [^{3}H]-thymidine was added to measure the increase in DNA synthesis, [^{3}H]-uridine was used to follow RNA synthesis and [^{3}H]-leucine gave a measure of protein synthesis. The cells were then harvested by using a cell harvester (Flow Laboratories, Irvine, Scotland) and the radioactivity precipitated by 10% (w/v) trichloroacetic acid was measured in a liquid scintillation spectrophotometer (Packard Tri-Carb, Packard Instrument Ltd; Reading, England).

The lymphocyte stimulation was expressed as log (CPM (stimulated) - CPM (control)) or as the Stimulation Index $\left(\frac{\text{CPM (stimulated)}}{\text{CPM (control)}}\right)$.

The control refers to the test sample incubated without mitogen.

2.2.5. Rat Spinal Cord Cell Culture

2.2.5.1. Preparation of Collagen-Coated Plates

The plates were coated with photopolymerised rat tail collagen according to the method of Masurousky and Peterson (1976). Culture dishes (3.5cm petri dishes, 24 well trays, and 10 well multi-spot slides) were coated with a collagen (1mg/ml)-riboflavin (0.05% w/v) mixture (ratio 4:1) which was spread evenly (10µl/cm^2) and then placed in an incubator at 37^{o}C overnight, and rinsed twice with sterile water to remove excess riboflavin. 30 min prior to the addition of the cells, the plates were incubated with Serum Supplemented Medium at 37^{o}C in an atmosphere of 10% CO_2, 90% air.

2.2.5.2. Preparation of a Single Cell Suspension from Rat Spinal Cord

Spinal cord cells were cultured according to the method outlined by Digby et al (1985) with minor modifications. Pregnant rats (15 day) were killed by cervical dislocation and the embryos (13-15) were removed under aseptic conditions. The spinal cords were dissected from the embryos and minced with irridectomy scissors in Puck's BSS (0.5ml). The cell suspension was then transferred to a round bottomed plastic test tube (12ml) by using the same buffer (3ml). Deoxyribonuclease I (1mg/ml) in H-Eagles medium (200µl) was added in order to prevent the cells becoming 'sticky' as a result of the release of nucleic acids; this hinders the subsequent formation of a single cell suspension. After 5 min at 20^{o}C, 0.5%

(w/v) trypsin in H-Eagles medium (200ul) was added. The cells were incubated at 37°C for 45 min with gentle mixing every 5 min then centrifuged for 5 min at 400xg. The supernatant was discarded and the spinal cord fragments were mechanically dissociated by trituration with a plastic Pasteur pipette by using samples (2ml) of SSM. The cell suspension ($3-5 \times 10^6$ /ml) was plated out on petri dishes at a density of 10^5 cells/cm^2 in culture medium. The cultures were then maintained in a humidified atmosphere containing 10% CO_2 at 37°C. After 3 days the culture medium was changed to serum-free medium and renewed every 3 days. From days 6-9 fluorodeoxyuridine (15ug/ml) and uridine (35ug/ml) were added daily. Addition of the inhibitor before this time led to excessive cell death.

2.2.6. Histochemical Staining

Coverslips were prepared as described in Section 2.2.5.1. and were fixed in 5% (w/v) acetic acid/ ethanol for 10 min at -20°C, or in (4%) paraformaldehyde. They were then washed four times with PBS until the pH was neutral, and incubated with either methylene blue (MB) or Delafeld's haematoxylin (HT) for 5-15 min (until the cells had stained slightly darker than desired) and washed with PBS. The coverslips were then incubated with 1% (w/v) eosin in water for 2 min and washed with PBS. All the coverslips (MB+HT) were dehydrated by incubation for 10 min with each of a series of ethanol-water mixtures containing 70%, 80%, 90%, 95%, and 100%

ethanol respectively. Finally the coverslips were incubated with xylene for 30 min and then mounted on a large microscope slide (2.5x8cm) with an organic solvent, DPX.

2.2.7. Immunocytochemical Identification of Cultured Spinal Cord Cells

Rat spinal cord cells were cultured on collagen-coated cover slips (13mm) in a similar manner to that described in section 2.2.5.1. The slides were washed twice with PBS (5ml each wash) and then fixed with 5% (v/v) acetic acid/ ethanol for 10 min at -20 °C. The cover slips were rinsed with PBS until the pH was neutral, and then incubated with 10% (v/v) rabbit serum in PBS for 30 min at room temperature. Mouse antibodies (anti-neurofilament, a kind gift from Adrian Rodgers and anti-tetanus toxin a kind gift from Ahmed Jehanli) in 10% (v/v) rabbit serum/PBS were added to the cover slips (100µl). After 30 min at room temperature, they were then washed by dipping ten times in 10% (v/v) rabbit serum/PBS. Finally the fixed cultures were incubated for 30 min in the dark at room temperature with a fluorescent-labelled rabbit anti-mouse IgG, diluted in 10% (v/v) rabbit serum/PBS. The cover slips were then finally washed as before and mounted on a large microscope slide (2.5x8cm) with 5% (v/v) n-propyl gallate in glycerol/PBS (9:1 v/v) pH 8.0 solution.

2.2.8. (a) Spinal Cord Membrane Preparation

The procedure of Snyder and Young (1973) was adapted to the neuronal cultures. Spinal cord cell cultures (15-16 day) were scraped by using a teflon-coated spatula from the petri dish surface (3.5cm, 20 dishes) in 0.32 M sucrose (0.5ml) and the cell suspension was then homogenised in a 1ml glass homogeniser for 10 min at 0 °C. The homogenate was centrifuged at 1000xg for 10 min and the resulting supernatant was then centrifuged at 15000xg for 30 min at 4 °C. The pellet was resuspended in 1mM Tris/HCl buffer, pH 8.0, (0.5ml) and homogenised for 10 min at 0 °C. The suspension was centrifuged at 9000xg for 20 min at 4 °C to give a supernatant that contained membrane fragments (0.5-0.6 mg/ml). Protein was estimated by a modification of the method of Lowry (Markwell et al, 1978).

2.2.8. (b) Spinal Cord Membrane Preparation

This procedure is identical to the above method but instead of cultured rat spinal cord cells, adult rat spinal cords were used. Rat spinal cords (20) were homogenised in 0.32M sucrose (10ml) for 10 min at 0 °C. The resulting homogenate was then centrifuged at 1000 x g for 10 min and the resulting supernatant was centrifuged at 15000 x g for 30 min at 4 °C. The pellet was resuspended in 1mM Tris/HCl buffer, pH 8.0, (10ml) and homogenised for 10 min at 0 °C. The suspension was centrifuged at 9000 x g for 20 min at 4 °C to give a supernatant that contained membrane fragments (3mg/ml).

This was referred to as a P3 membrane fraction. The protein content of the supernatant was estimated as described above.

2.2.9. Monoclonal Antibody Procedures

2.2.9.1. Myeloma Cell Line Maintenance

P3X63.Ag8-653, a mouse myeloma B-cell line, was kept under continuous culture in cloning medium (see section 2.1.6. (c)). The cells were maintained at a density of 0.2-0.8 x10^6 /ml, being supplemented with fresh medium every 3 days.

2.2.9.2 Macrophage Preparation

Balb-c mice (4 weeks old) were killed by cervical dislocation and placed in 75% (v/v) ethanol in water. The mice were removed from the liquid, the skin was dissected away from over the liver and PBS (5ml) was injected into the peritoneal cavity by using a 5ml syringe with an 18g needle. The mice were then rocked for 2 min. The PBS was removed by drawing the liquid back into the 5ml syringe with a 28g needle, and placed in a 5ml Bijou bottle. The cell suspension was irradiated with ^{45}Co (400 rads, 4 min) and the cells were then counted by using a haemocytometer. The macrophages were centrifuged at 400xg for 10 min at room temperature and resuspended in cloning medium at 1x10^6 /ml. They were then distributed (50µl) to each well of a 96 well flat bottomed microtitre tray, and incubated for 24h at 37 ^{o}C in a 5% humidified atmosphere of CO_2 in air prior to use.

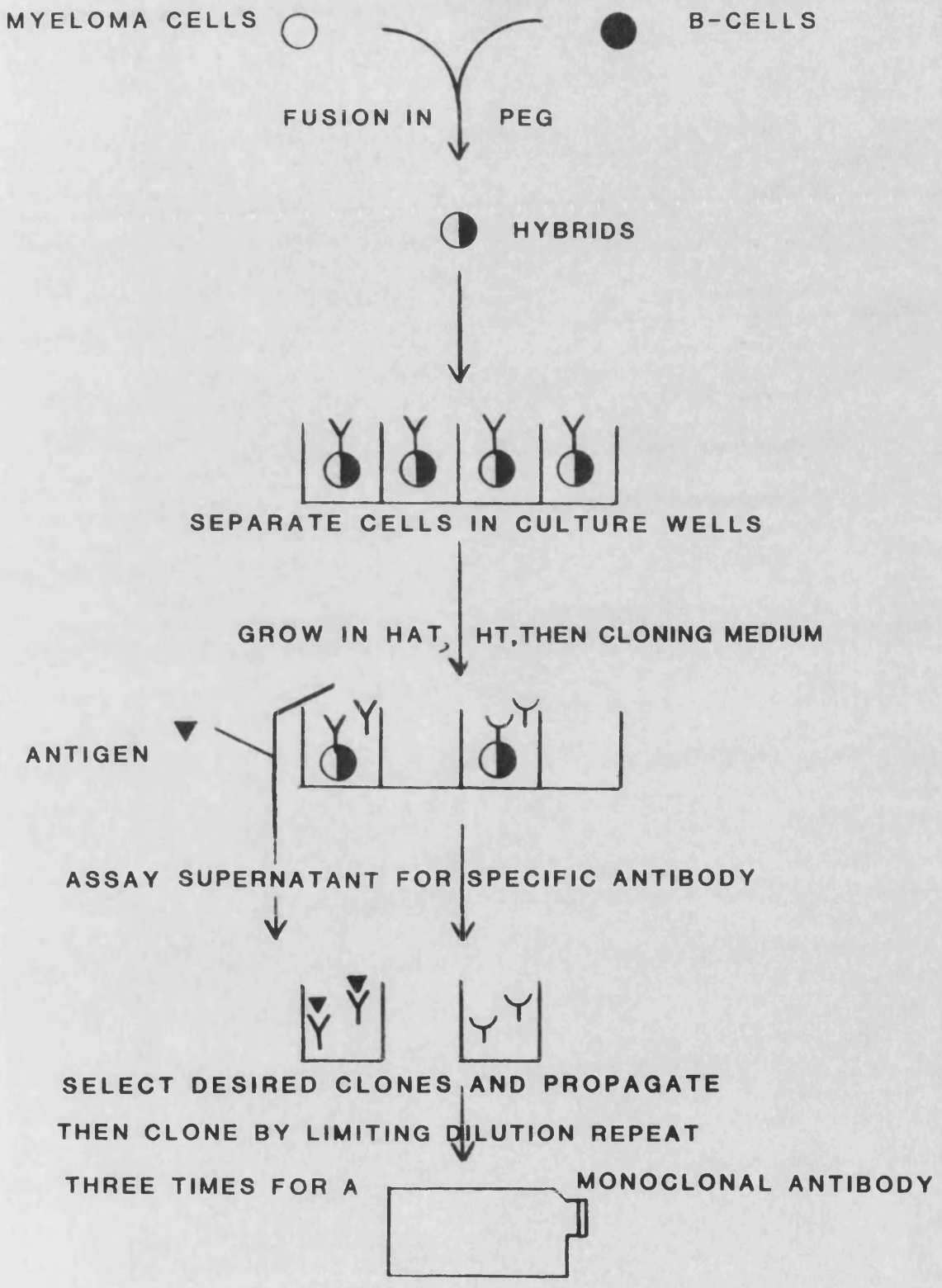
2.2.9.3 Cell Fusion

Lymphocytes were separated from fresh venous blood (see Section 2.2.1.(a)) and mixed with the P3X63.Ag8-653 mouse myeloma cell line in the ratio of 2:1. The lymphocytes and the Ag8 cells were centrifuged to a common pellet at 200xg, for 10 min at room temperature in a 50 ml siliconised glass centrifuge tube. The supernatant was discarded and the pellet was resuspended in RPMI 1640 and centrifuged again at 200xg for 10 min at room temperature. The supernatant was discarded and the pellet was loosened by tapping the centrifuge tube gently. The centrifuge tube was then placed in a water bath at 37 °C, and 50% (w/v) polyethylene glycol 6000 (1 ml) was added over a period of 1 min after which the suspension was swirled for 90 sec in a water bath at 37 °C. The centrifuge tube was then removed from the water bath and RPMI 1640 (1 ml) was added over 1 min; this operation was then repeated. RPMI 1640 (2 ml) at 37 °C was then added over 30 sec, and this was repeated. Finally, RPMI 1640 (7 ml) was added over 2 min and the volume was made up to 20 ml. The cell suspension was then allowed to stand for 10 min at room temperature, after which the tube was centrifuged at 200xg, for 10 min at room temperature. The supernatant was discarded and the pellet carefully resuspended (1x10 6 Ag 8/ ml) in cloning medium containing HAT. The cells were then distributed at a density of 200,000 cells/well of a 96 well flat bottomed tray already preseeded with macrophages. The medium was changed by removing an aliquot (100µl) with a multipipetter

and replenishing with fresh cloning medium plus HAT at 37°C for 4 weeks . The medium was changed to cloning medium plus HT, for a period of 2 weeks after which cloning medium alone was added.

FIGURE 2.1 General Procedures Followed for the Production of Monoclonal Antibodies

Mouse myeloma cells were fused with B-cells using polyethylene glycol and the hybrids plated out in culture wells. These hybrids were grown in HAT medium for 2 weeks, then HT medium and finally cloning medium. This procedure eliminates any unfused cells allowing hybrids to grow. The desired colonies were then selected, propagated and then cloned by limiting dilution. This procedure was repeated 3 times to obtain a monoclonal antibody.


MYELOMA CELLS
B-CELLS
FUSION IN PEG
HYBRIDS
SEPARATE CELLS IN CULTURE WELLS
GROW IN HAT, HT, THEN CLONING MEDIUM
ANTIGEN
ASSAY SUPERNATANT FOR SPECIFIC ANTIBODY
SELECT DESIRED CLONES AND PROPAGATE
THEN CLONE BY LIMITING DILUTION REPEAT
THREE TIMES FOR A
MONOCLONAL ANTIBODY

2.2.10. Epstein-Barr Virus Transformation

2.2.10.1. The Production of EBV-containing supernatant

The EBV-containing supernatant was prepared by using the method of Haskard and Archer (1984). Marmoset blood leukocytes (B95-8) were cultured in 10% FCS/RPMI 1640 containing 2mM pyruvate for 10 days at a starting cell concentration of 10^5 cells /ml in T flask at 37 °C. The Epstein-Barr virus is released extracellularly into the culture fluid, which is centrifuged at 400xg at 4 °C for 10 min. The supernatant was then filtered through a 0.45µm filter and stored at -70 °C.

2.2.10.2. EBV Transformation of Human Lymphocytes

Peripheral blood lymphocytes were cultured initially in flasks with Pokeweed Mitogen (20µg/ml/$1x10^6$ cells) for 5 days. The cells were counted, washed twice with RPMI 1640 and centrifuged at 200xg for 10 min at room temperature in 30ml sterilin tubes. The pellet was loosened by gentle tapping and the EBV supernatant was added at 1ml/$3x10^6$ cells for 1 h, and the tubes were incubated at 37 °C in an atmosphere of 5% CO_2 in air.

After 1 h the cells were washed twice by centrifuging at 200xg for 10 min at room temperature in RPMI 1640. Finally the pellet was resuspended in cloning medium at a concentration of 0.5 $x10^6$ cells/ml. The suspension was then dispensed (200µl) to each well of a 96 well flat bottomed tray already preseeded with macrophages.

The supernatant was replenished as previously described.

2.2.10.3. Cloning by Limiting Dilution

Antibody-secreting clones must be recloned as soon as possible and in order to become stable must be recloned at least 3 times. This was performed by plating out cells at 1, 2, and 5 cells/well on 96 well flat bottomed microtitre tray, previously preseeded with macrophages. The cells were then incubated at 37 ^{o}C in an atmosphere of 5% CO_2 in air until clones appeared, the medium being replenished every 7 days. Once clones appeared the supernatants were assayed for the desired antibody and the appropriate clone(s) were grown up in 24 well trays.

2.10.4 Storage of Clones in Liquid Nitrogen

The cells were centrifuged at 200xg for 10 min at room temperature. The supernatant was discarded and the cells were washed twice with RPMI 1640 at 37 ^{o}C, counted and resuspended in 50% (v/v) Fetal Calf Serum-RPMI 1640 at $10x10^6$ cells/ml. 20% (v/v) DMSO-RPMI 1640 was added dropwise to bring the concentration to $5x10^6$ cells/ml. The cells were then put in a freezer at -70 ^{o}C overnight and then transferred to liquid nitrogen (-196 ^{o}C) for storage.

2.2.11 Partial Purification of Immunoglobulins by Salt Fractionation

Culture supernatant (50ml) was warmed to 25 °C and sodium sulphate (9g) was added with stirring to make an 18% (w/v) solution. The solution was stirred at 25 °C for 30 min, and centrifuged at 3000xg for 30 min at 25 °C. The supernatant was discarded. The precipitate was then redissolved in distilled water up to a maximum of 25ml and the solution was dialysed against PBS.

2.2.12. Detection of Immunoglobulin Production

2.2.12.12 Immuno-dot binding assay

All steps in the dot-immunobinding assay were done at room temperature unless otherwise stated, and all incubations were done on a shaking platform to provide gentle agitation.

Nitrocellulose membrane was cut into strips of convenient size and labelled with waterproof ink for identification. After soaking in 50 mM Tris/HCl, 0.15M NaCl, pH 7.4 buffer containing 0.01% thiomersal (TBS), the strips were dried (tweezers were used at all times to handle the strips). Serial dilutions of anti-human IgG, IgM, IgA, or anti-human light chains (1µl) from 1µg/ml - 50ng/ml in TBS were spotted on the sheet. The strips were then allowed to dry, and were incubated for 1 h with 1% (w/v) casein in TBS to block any unoccupied sites. The nitrocellulose sheet was then incubated with human monoclonal supernatant overnight at 4 °C. The nitrocellulose sheet was then washed three times with 1%(w/v)

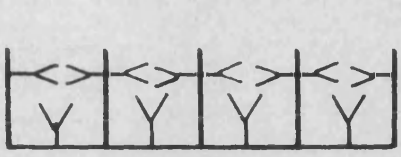
casein in TBS, each for 5 min at room temperature. Horse-radish peroxidase conjugated goat anti human IgG, IgM or IgA antisera (diluted 1:1000) was then added and incubated for 2 h. The nitrocellulose sheet was finally washed 3 times with 1% (w/v) casein in TBS and incubated with the substrate, 0.02% (w/v) 3-amino-9-ethyl carbazole and 0.03% H_2O_2 in 50mM sodium acetate buffer, pH 5.0, for 15 min. When "spots" appeared the substrate, was removed and the sheet was then washed five times with water and then dried by using filter paper. The control "spots" were normal human IgG, IgM, or IgA (1µg/ml).

2.2.12.2. Competitive Enzyme Linked Immunoassay (CELIA)

To determine immunoglobulin class and concentration in the human monoclonal supernatants, a CELIA was established (see Fig 2.2). Microtitre plates were coated overnight at $4^{o}C$ with human IgG, IgM, or IgA (2oong/ml) in 50mM sodium bicarbonate buffer, pH 9.6. Serial dilutions of the standard immunoglobulins from 10^{5} ng/ml to 10ng/ml or appropriately diluted culture

FIGURE 2.2 Competitive Enzyme LInked Immunoassay

The specific antibody was attached to the solid phase and then washed. The solid phase was then blocked to fill any unoccupied antibody sites. During this procedure standard dilutions of the specific antibody or unkown antibody concentration were incubated with enzyme-conjugated anti-antibody in separate tubes. The solid phase was then washed and the solutions from the tubes added. Competition then takes place between the solid phase-coupled antibody and the free standard antibody or the unknown antibody for the enzyme-conjugated anti-antibody. The solid phase was then washed and the enzyme-labelled substrate added. The inhibition of the colour change is proportional to the amount of antibody present.



Coated overnight 4° C

IgG ,IgM, or IgA (200ng/ml)

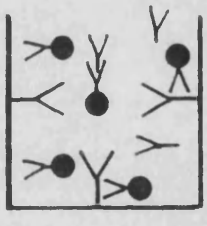
WASH 3 TIMES PBS-TWEEN

Incubate 30 min 1% (w/v) casein

Standard Ig dilutions or culture supernatants and the alkaline phosphatase conjugate

WASH 3 TIMES PBS-TWEEN

Add solution to cuvettes 1h



Competition between bound Ig and free standard Ig for the alkaline phosphatase conjugate

WASH 3 TIMES PBS-TWEEN

Add substrate for 1h

● :ALKALINE PHOSPHATASE ANTI-IMMUNOGLOBULIN ANTIBODY

supernatants in 0.05% (v/v) tween-PBS were transferred to LP3 tubes containing an equal volume of the appropriate alkaline phosphatase anti-immunoglobulin conjugate (diluted 1:1000 with 0.05% (v/v) PBS-tween) and incubated for 1 h at 37°C. Meanwhile, the coated cuvettes were washed three times, each for 5 min with PBS-tween and then incubated with 1% (w/v) casein in PBS for 30 min to block any unoccupied sites. Then the mixture (250µl) in the LP3 tubes was transferred to the now blocked cuvettes and incubated for 1 h at 37°C. The cuvettes were washed three times, each for 5 min with 0.05% (v/v) tween-PBS and the appropriate substrate added. Para nitro-phenyl phosphate (PNPP) (1mg/ml) was the substrate used for alkaline phosphatase and was added in 0.1M glycine buffer,pH 10.4, with 1mM $MgCl_2$ and 1mM $ZnCl_2$ for 1 h. The reaction was stopped by adding 1M NaOH and the absorbance was read at 405 nm. The inhibition of the colour change being proportional to the amount of antibody . The assay is summarised in Fig. 2.2.

2.2.12.3. Double Antibody Sandwich Enzyme-Linked Immunoabsorbent Assay

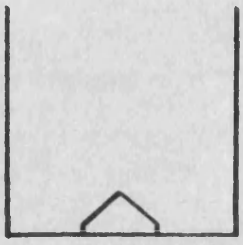
Microtitre plates were coated with sheep anti-human light chain antibodies (supplied by Dr. A. Jehanli of this department), (200ng/ml; 100µl) in 50mM sodium bicarbonate buffer, pH 9.6, overnight at 4°C. The plates were then washed with 0.2% (w/v) casein in 0.05% (v/v) tween-PBS (250 µl) buffer three times for 5 min each, and then incubated with the buffered casein for 30 min

at room temperature to block any unoccupied sites. The plates were washed and the various dilutions of standard solutions of IgG, IgM, or IgA in the 0.2% (w/v) casein buffer containing 2.5% (v/v) normal goat serum (100µl) were added and incubated overnight at 4 °C. Again the plates were washed three times with the buffer and the horse-radish peroxidase-goat anti human IgG, IgM, or IgA was added (diluted 1:500 with casein buffer plus 2.5% (v/v) normal goat serum) (100µl) for 3 h at room temperature. This was then removed and the trays washed three times for 5 min with the casein buffer with one last wash with PBS. The wells were then incubated with the substrate for the HRPO which is 3,3'-5-5'-tetramethyl benzidine (TMB) at a final concentration 42mM made in 0.1M sodium acetate / citric acid buffer, pH 6.0, containing 0.004% (v/v) H_2O_2 (100µl) 30 min -1h at room temperature. An intense blue colour developed E max = 655 nm,and the reaction was stopped by the addition 2M sulphuric acid (20µl). The assay is sumarised in Fig 2.3

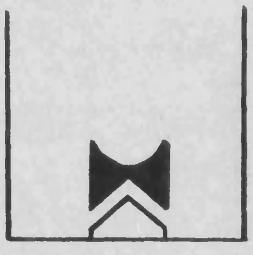
FIGURE 2.3 Double Antibody Sandwich ELISA

Antibodies were attached to the solid phase which was then washed. The test solution was then incubated with the solid phase and then washed. Enzyme-labelled specific antibody to the unknown antibody was then incubated with the solid phase followed by washing. The enzyme substrate was then added. The colour change being proportional to the amount of unknown antibody in the test solution.

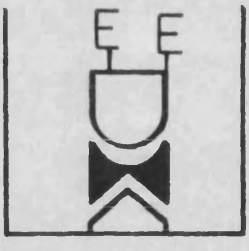
1) SHEEP ANTI-HUMAN LIGHT CHAIN ANTIBODIES



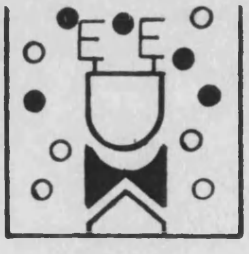
2) TEST SOLUTIONS (KNOWN DILUTIONS OF STANDARD IMMUNOGLOBULINS OR CULTURE SUPERNATANTS)



3) ENZYME-LABELLED GOAT ANTI-HUMAN IMMUNOGLOBULIN ANTIBODIES



4) ENZYME SUBSTRATE ADDED



2.2.12.4. Substrates for horse radish peroxidase

(a) O-phenylenediamine (OPD): Hydrogen peroxide (0.012% v/v) was added just before use to a freshly prepared solution of OPD (0.4 mg/ml) in 0.1M citric acid/0.2M Disodium phosphate buffer, pH 5.0. The reaction stopped by the addition of sulphuric acid (2M,100µl). Optical density was read at 494nm.

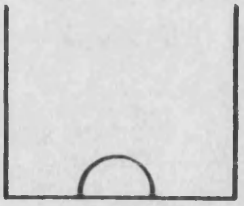
(b) 3,3',5,5'-tetramethylbenzidine (TMB) : The TMB was added at a final concentration 42mM made in a sodium acetate/ citric acid buffer, pH 6.0, containing 0.004% (v/v) H_2O_2 (100µl) for 30 min-1h at room temperature. The reaction was stopped by the addition of 2M sulphuric acid (20µl).

2.2.12.5. ELISA for Anti-Neuronal Membrane Antibodies

Microtitre plates were coated with neural membrane preparation P3 (see section 2.2.8. (b)) (5µg/ml) in 50mM sodium bicarbonate buffer, pH 9.6, overnight at 4 °C. The plates were then washed with 0.2% (w/v) casein in 0.05% (v/v) tween-PBS buffer three times for 5 min each, and then incubated with the buffered casein for 30 min at room temperature to block any unoccupied sites. The plates were washed and the culture supernatants from the clones were added (100µl) and allowed to stand for 2 h at room temperature. Again the plates were washed three times with the buffer and the alkaline phosphatase anti-human whole immunoglobulin (contains IgG, IgM, and IgA) (100ul) was added (diluted 1:500 with casein buffer plus 2.5% (v/v) normal goat serum) for 2 h at room temperature . T

removed and the trays were washed three times for 5 min with casein buffer and finally with PBS. The wells were then incubated with the substrate PNPP for 1 h at 37 °C. An intense colour developed and the reaction was stopped by adding 1M NaOH. The absorbance was read at 405nm. Fig 2.4 summarises this assay.

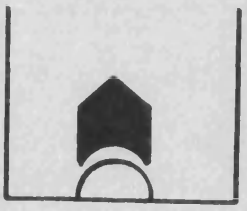
FIGURE 2.4 ELISA for the Detection of Anti-Neuronal Membrane Antibodies



P3 MEMBRANE FRACTION

ADSORBED TO PLATE

WASH

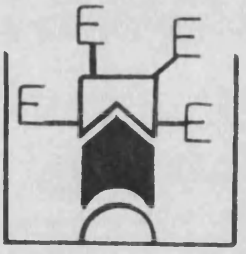


CULTURE SUPERNATANTS

:ANY SPECIFIC ANTIBODY

ATTACHES TO ANTIGEN

WASH

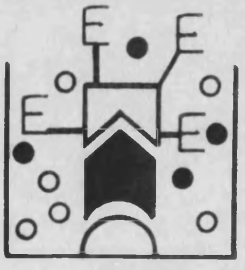


ENZYME-LABELLED

ANTIGLOBULIN ATTACHES

TO ANTIBODY

WASH



ENZYME SUBSTRATE ADDED

CHAPTER 3 RESULTS

SECTION 3.1 ~ LYMPHOCYTE STIMULATION STUDIES

3.1.1. Lymphocyte Preparation

The numbers of lymphocytes recovered after separation on a ficol-hypaque gradient varied depending upon whether whole blood or plasma-deficient blood was used, the age of the blood sample and whether blood stored at room temperature or 4°C were used.

The blood samples were drawn from the patient in the morning. The blood was stored overnight, either at room temperature or 4°C, or used fresh. Lymphocyte numbers from fresh blood and from that held overnight at room temperature were essentially the same. Storage of the blood for 24 h at 4°C resulted in the number of lymphocytes being drastically reduced (by as much as 75%). If the blood was stored at room temperature for longer than 24 h the numbers of lymphocytes steadily declined (~20%/day) until, after 4 days, very few lymphocytes could be recovered.

Larger numbers of lymphocytes from a ficol-hypaque gradient were consistently obtained from whole blood compared with blood from which the plasma had been removed (~50% less) by centrifugation. Figure 3.1 shows a gradient before and after centrifuging at 400 x g for 35 min at room temperature. The "fluffy" white band at the interface contains the lymphocytes. Using whole blood, this band is approximately twice as thick as the plasma deficient blood sample. Contamination of the band with

red blood cells was also less when whole blood was used.

3.1.2. Blastogenic Activity of Fresh and Frozen Lymphocytes

Lymphocytes were separated from 5 samples (20 ml, each) of venous blood. Each sample was divided into four equal aliquots, three of which were kept in liquid nitrogen (-196^{o}C). The fourth was stimulated with Concanavalin A (Con A) and with Phytohaemagglutinin (PHA) (7.5µg/ml) for 3 days. After one month, one aliquot of each frozen lymphocyte sample was chosen, rapidly thawed and stimulated with Con A and PHA. This was then repeated after two and three months.

The stimulation was measured by using five assays which included [^{3}H]-thymidine, [^{3}H]-uridine, and [^{3}H]-leucine incorporation into DNA, RNA, and protein respectively; glucose consumption, and lactate release. The latter assays were used as measures of metabolic activity. The results in Tables 3.1 (a) and 3.1 (b) show that the ability of lymphocytes to undergo blastogenesis was progressively reduced by freezing.

TABLE 3.1 (a) The Blastogenic Activity by Concanavalin A of Fresh and Frozen Lymphocytes

	STORAGE CONDITIONS			
	FRESH	1 MONTH FROZEN	2 MONTH FROZEN	3 MONTH FROZEN
^{3}[H]-Thymidine Incorporation Log cpm	4.04 +_0.11	3.41 +_0.21	2.63 +_0.14	2.05 +_0.07
^{3}[H]-Uridine Incorporation Log cpm	3.84 +_0.08	3.10 +_0.12	2.50 +_0.12	1.30 +_0.04
^{3}[H]-Leucine Incorporation Log cpm	4.0 +_0.22	3.25 +_0.14	2.50 +_0.11	2.00 +_0.05
Lactate Released µg/ml	859 +_124	654 +_121	432 +_111	249 +_104
Glucose Consumed µg/ml	0.81 +_0.11	0.65 +_0.09	0.35 +_0.14	0.15 +_0.04

Stimulation Index Mean +_ Standard Deviation

Number of experiments = 4

TABLE 3.1 (b) The Blastogenic Activity by Phytoheamagglutinin of Fresh and Frozen Lymphocytes

	STORAGE CONDITIONS			
	FRESH	1 MONTH FROZEN	2 MONTH FROZEN	3 MONTH FROZEN
^{3}[H]-Thymidine Incorporation Log cpm	4.17 +_0.15	3.51 +_0.10	3.01 +_0.11	2.50 +_0.21
^{3}[H]-Uridine Incorporation Log cpm	3.94 +_0.30	2.98 +_0.11	2.05 +_0.12	1.15 +_0.10
^{3}[H]-Leucine Incorporation Log cpm	4.12 +_0.21	3.55 +_0.16	2.15 +_0.14	1.95 +_0.10
Lactate Release µg/ml	956 +_129	715 +_50	441 +_121	214 +_111
Glucose Consumed µg/ml	0.64 +_0.11	0.41 +_0.11	0.34 +_0.05	0.21 +_0.04

Stimulation Index Mean +_ Standard Deviation

No. of experiments =4

3.1.3. Glucose Assay with Lymphocyte Culture Medium

The glucose assay was performed on lymphocyte culture medium (100-0% v/v PBS) which had been treated by exactly the same method as patient stimulation assays (see Section 2.2.4. (a)). The medium was diluted with PBS.

The results shown in Fig. 3.2 indicate that the glucose assay accurately detects the amount of glucose in the medium. The theoretical value of the medium (100% v/v) was 2mg/ml.

3.1.4. Dose-Response Study of Blastogenesis

Lymphocytes ($1x10^6$) separated as described in Section 2.2.3 from 5 normal controls (age range 23-65 years : mean 52 yrs) were stimulated with increasing concentrations of Con A, and PHA (0-20µg/ml) for 3 days and incubated in a humidified atmosphere of 5% CO_2 in air at 37°C.

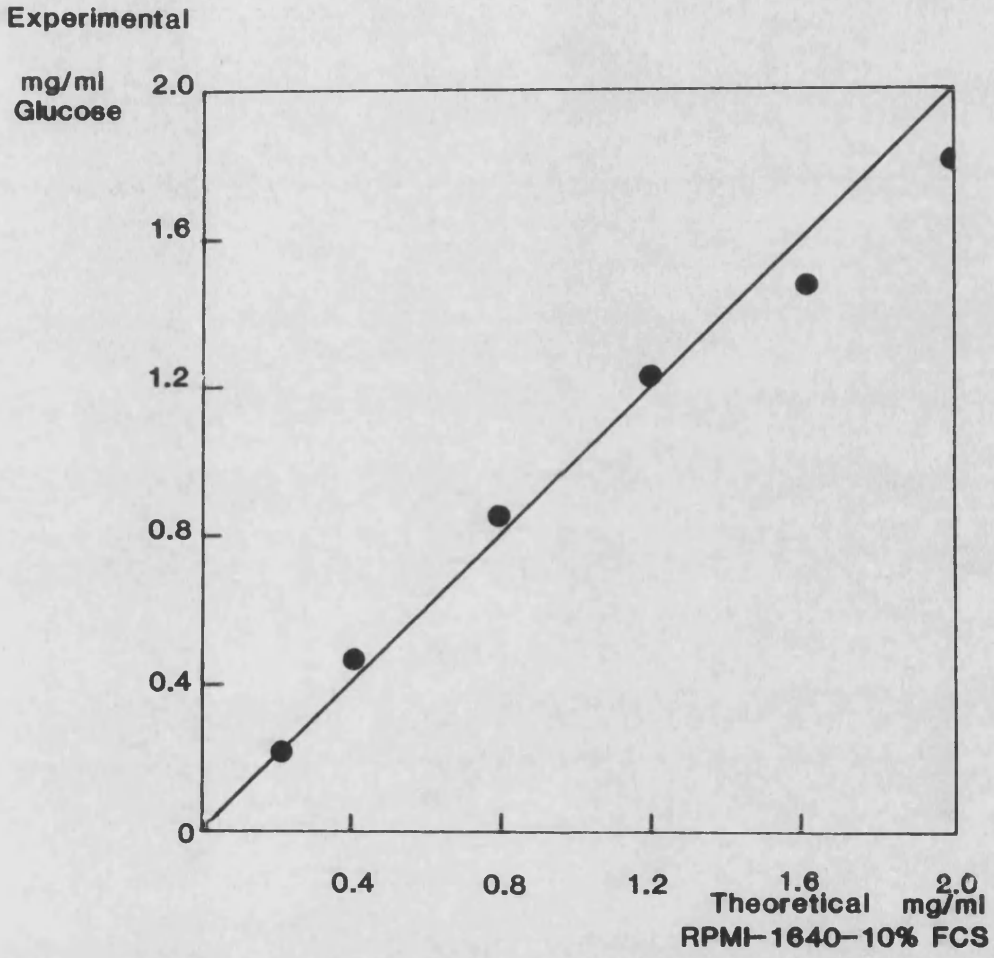
Stimulation of lymphocytes was assayed by following the incorportion of radiolabelled thymidine, uridine, and leucine into DNA, RNA, and protein repectively. Lactate production and glucose consumed were also measured as indices of metabolic activity.

The results (Fig. 3.3) show that maximum response was observed in all 5 assays with 10-15µg/ml mitogen. In all following procedures 7.5µg/ml mitogen was used.

3.1.5. Time-Response Study of Blastogenesis

Lymphocytes were separated from 5 normal donors (age range 23-65yr : mean 51 yr) and incubated for a period of from one to seven days with Con A and PHA as described in Section 2.2.1. (a). The response was measured by the incorporation of radiolabelled precursors, thymidine, uridine, and leucine into DNA, RNA, and protein repectively. Lactate released and glucose consumed were also measured. The results, shown in Fig. 3.4, reveal that the optimum period was 2-4 days for incorporation of radiolabelled precursors. Lactate release also reached a peak between 2 and 4 days. Glucose consumption increased rapidly for the first 3 days and then assumed a steady level.

FIGURE 3.2 The Glucose Assay with Lymphocyte Culture Medium


Experimental
mg/ml
Glucose
2.0
1.6
1.2
0.8
0.4
0
0.4
0.8
1.2
1.6
2.0
Theoretical mg/ml
RPMI-1640-10% FCS

S.D. 5%

FIGURE 3.3 The Dose-Response Study of Blastogenesis by Con A and PHA

The effect of concentration of PHA and Concanavalin A on the stimulation of normal human lymphocytes. Values, obtained after 3 days in culture as described in the Materials and Methods Section 2.2.4., are the means from 5 separate determinations using lymphocytes from different normal individuals and are expressed as the difference (in cpm or μg/ml) between test sample and that incubated in the absence of mitogen.

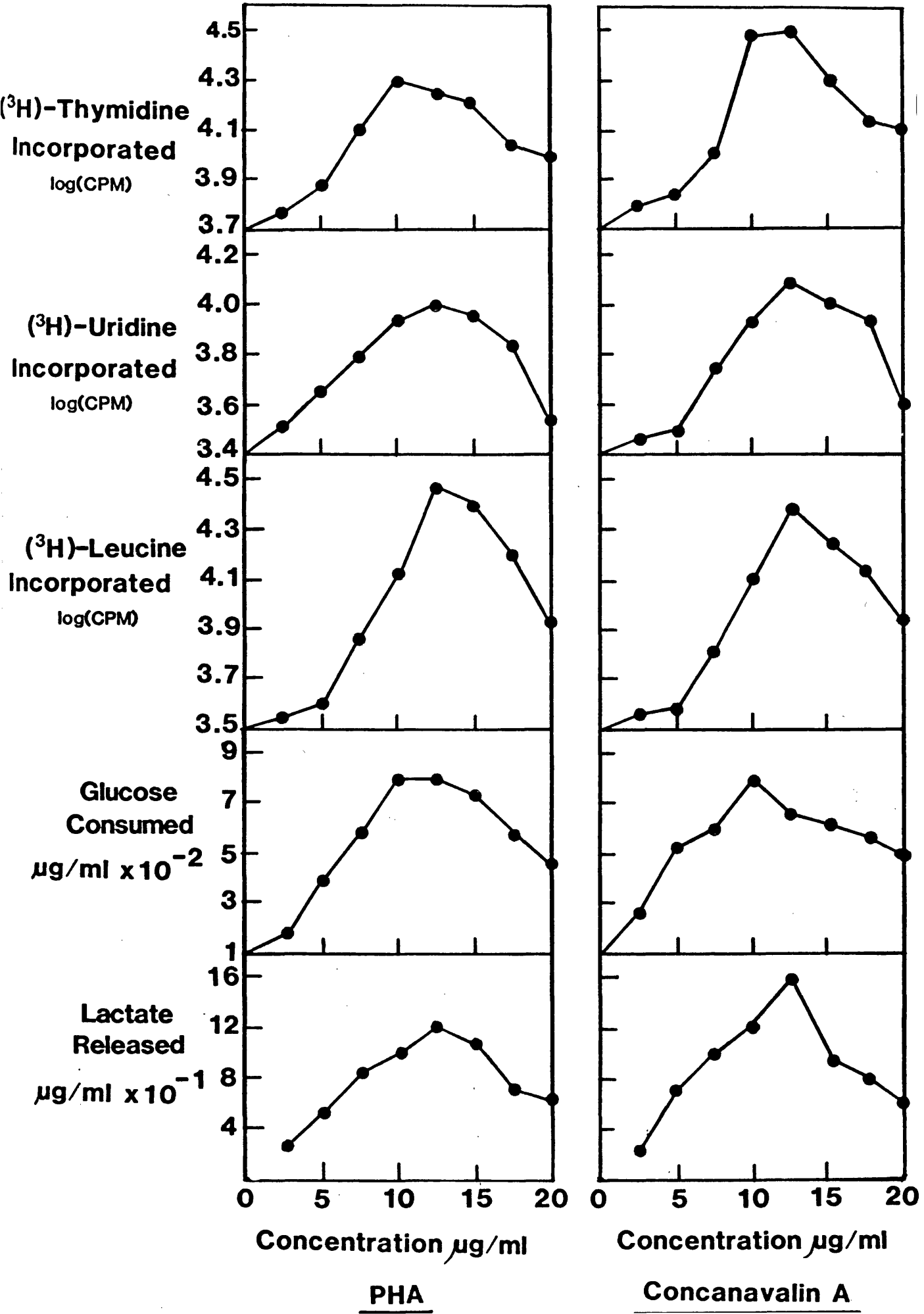
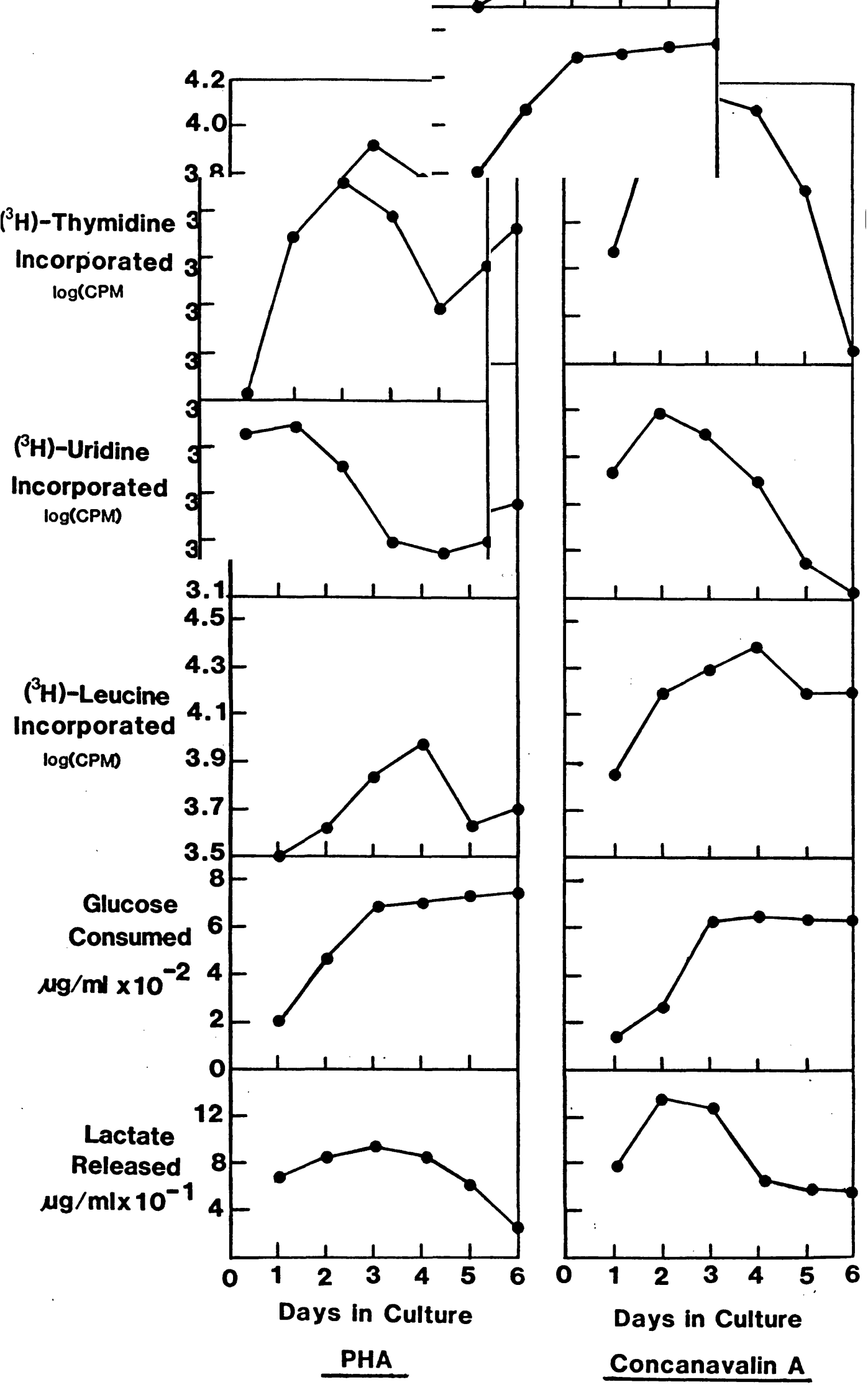

(3H)-Thymidine Incorporated log(CPM)
4.5
4.3
4.1
3.9
3.7
(3H)-Uridine Incorporated log(CPM)
4.2
4.0
3.8
3.6
3.4
(3H)-Leucine Incorporated log(CPM)
4.5
4.3
4.1
3.9
3.7
3.5
Glucose Consumed µg/ml x10-2
9
7
5
3
1
Lactate Released µg/ml x10-1
16
12
8
4
0
5
10
15
20
Concentration µg/ml
PHA
Concanavalin A

FIGURE 3.4 The Time-Response Study of Blastogenesis with Con A and PHA

The effect of time on the stimulation of normal human lymphocytes by PHA and Concanavalin A. Values obtained by using 7.5 µg/ml mitogen as described in the Materials and Methods Section 2.2.3., are the means of five separate experiments using lymphocytes from different normal individuals and are expressed as the difference (in cpm or µg/ml) between test sample and that incubated in the absence of mitogen.


(3H)-Thymidine Incorporated log(CPM
(3H)-Uridine Incorporated log(CPM)
(3H)-Leucine Incorporated log(CPM)
Glucose Consumed µg/ml x10-2
Lactate Released µg/ml x10-1
Days in Culture
PHA
Days in Culture
Concanavalin A

3.1.6. Stimulation by Con A and PHA of Lymphocytes from Motor Neurone Diseased Patients and Normal Healthy Controls

Peripheral blood lymphocytes from 18 MND patients (age range 40-80 yrs : mean 61.1 +_ S.D. 9.8 yr) and from 20 age and sex matched controls (age range 41-77 yr : mean 55.6 +_ 9 yr) were stimulated by using sub-optimum concentrations of Con A and PHA (7.5µg/ml) for the optimum period of incubation (3 days). The results are shown in Table 3.2 and Figures 3.5 (a) and (b).

The incorporation of [^{3}H]-thymidine, and [^{3}H]-leucine and the consumption of glucose by MND lymphocytes was in each case significantly depressed compared with the age and sex -matched controls ($p<0.01$) with both mitogens. In the case of [^{3}H]-uridine incorporation and lactate release there was no significant difference between the MND lymphocytes and those from the age-matched controls.

TABLE 3.2 Stimulation by Con A and PHA of Lymphocytes from Patients with MND and from Normal Controls

(A) Con A

	Normal Controls (20) Mean +_ SD	MND Patients (18) Mean +_ SD	Significance (P = 0.01)
^{3}[H]-Thy (log CPM)	4.13+_0.19	3.69 +_ 0.34	S
^{3}[H]-Uri (log CPM)	3.83 +_0.31	3.78 +_ 0.33	NS
^{3}[H]-Leu (log CPM)	3.91 +_0.30	3.36 +_ 0.67	S
Lactate Prod: (µg/ml)	200 +_ 94	191 +_ 142	NS
Glucose Cons: (µg/ml)	453 +_ 221	286 +_ 145	S

(B) PHA

	Normal Controls (20) Mean +_ SD	MND Patients (18) Mean +_ SD	Significance (P=0.01)
^{3}[H]-Thy Uptake (log CPM)	3.94 +_ 0.23	3.35 +_ 0.38	S
^{3}[H]-Uri Uptake (log CPM)	3.74 +_ 0.32	3.56 +_ 0.32	NS
^{3}[H]-Leu Uptake (log (CPM)	3.93 +_ 0.32	3.57 +_ 0.39	S
Lactate Rel (µg/ml)	142 +_125	141 +_ 83	NS
Glucose Cons: (µg/ml)	579 +_ 224	382 +_ 212	S

3.1.7. Stimulation of MND Lymphocytes with Rat Spinal Cord Membranes : Optimisation of Membrane Protein Concentration

Lymphocytes from MND patients were incubated with increasing concentrations of P3 membrane fragments (0-300µg protein /ml) as determined by Lowry et al (1959). This was prepared as described in Section 2.2.8. (a) from 15-17 day old cultures of rat spinal cord cells which at this time consist predominantly of large clusters. Lymphocyte proliferation was assayed by the incorporation of labelled leucine and thymidine.

The results, shown in Fig. 3.6, indicate a maximum uptake of radiolabel up to 100µg/ml membrane protein, following which the uptake declined. For subsequent assays, a suboptimal dose of 50µg/ml was used.

FIGURE 3.5 (a) Stimulation By Con A of Lymphocytes from Motor Neurone Diseased Patients and Normal Healthy Controls

Values, obtained as described in the Materials and Methods Section 2.2.4., are the means of triplicate assays of lymphocytes from each individual donor and are expressed as the difference (in cpm or μg/ml) between test sample and that incubated in the absence of mitogen.

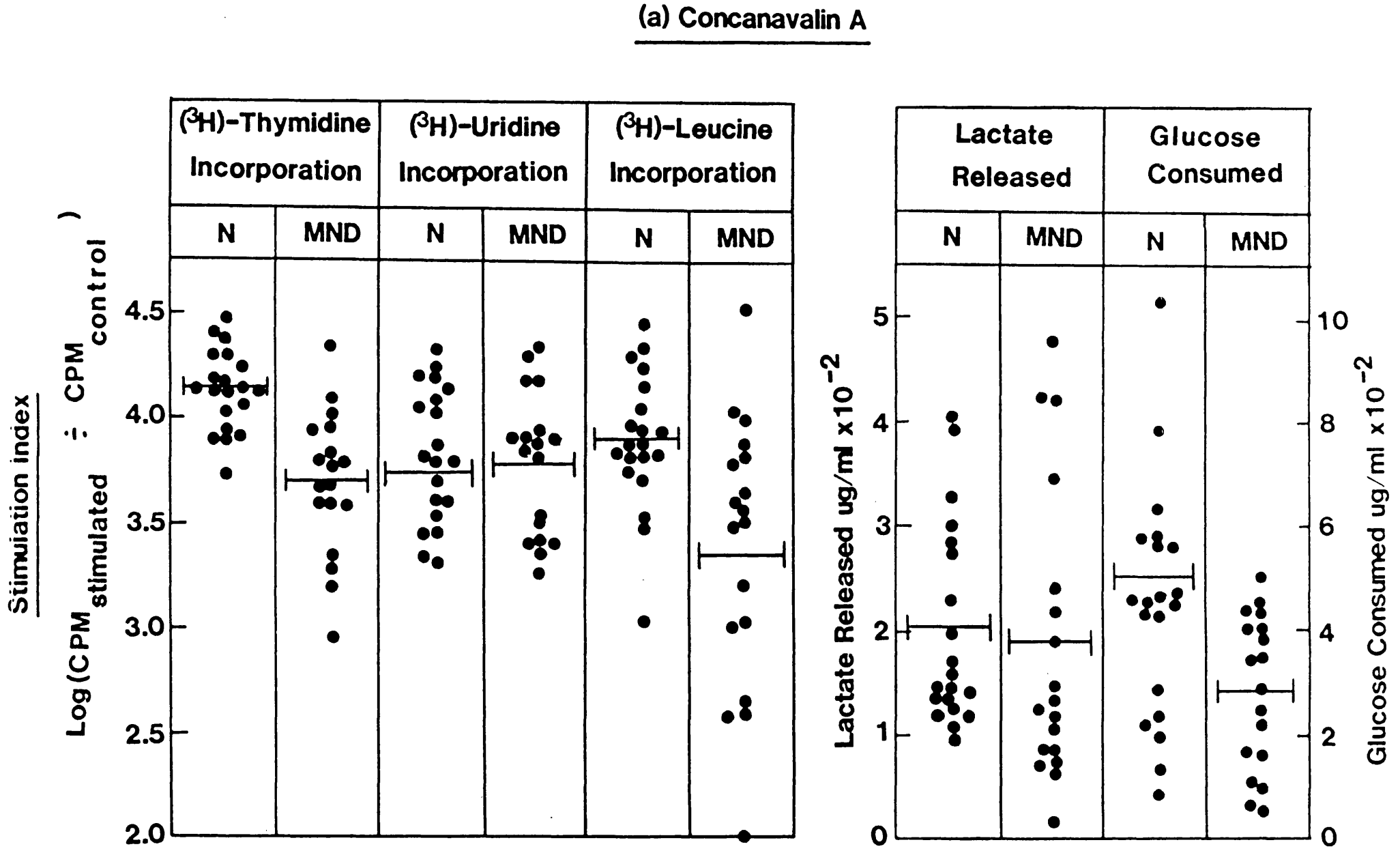

(a) Concanavalin A
Stimulation index
Log (CPM stimulated ÷ CPM control)
(3H)-Thymidine Incorporation
(3H)-Uridine Incorporation
(3H)-Leucine Incorporation
N
MND
N
MND
N
MND
4.5
4.0
3.5
3.0
2.5
2.0
Lactate Released
Glucose Consumed
N
MND
N
MND
Lactate Released ug/ml x10-2
Glucose Consumed ug/ml x10-2
5
4
3
2
1
0
10
8
6
4
2
0

FIGURE 3.5 (b) Stimulation By PHA of Lymphocytes from Motor Neurone Diseased Patients and Normal Healthy Controls

Values, obtained as described in the Materials and Methods Section 2.2.4., are the means of triplicate assays of lymphocytes from each individual donor and are expressed as the difference (in cpm or μg/ml) between test sample and that incubated in the absence of mitogen.

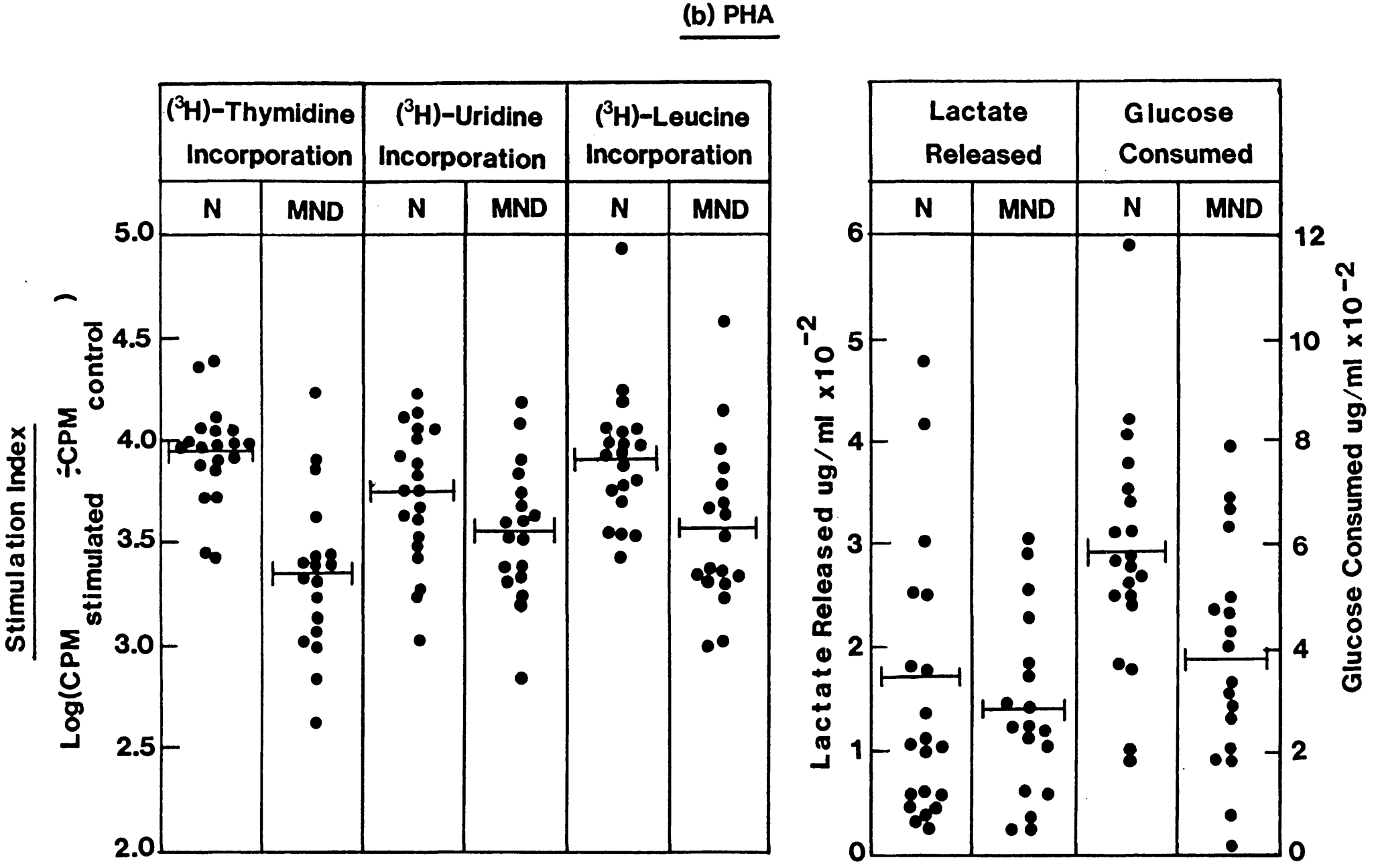
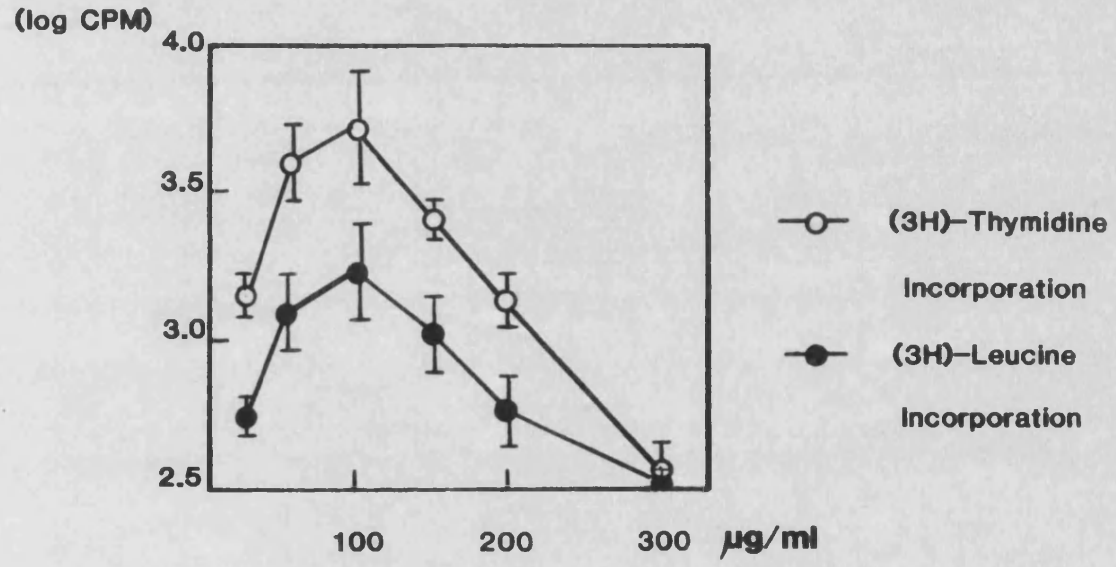

(b) PHA
Stimulation Index
Log(CPM stimulated ÷ CPM control)
(3H)-Thymidine Incorporation
(3H)-Uridine Incorporation
(3H)-Leucine Incorporation
N
MND
N
MND
N
MND
5.0
4.5
4.0
3.5
3.0
2.5
2.0
Lactate Released
Glucose Consumed
N
MND
N
MND
Lactate Released ug/ml x10-2
Glucose Consumed ug/ml x10-2
6
5
4
3
2
1
0
12
10
8
6
4
2
0

FIGURE 3.6 The Dose-Response Study of MND Lymphocytes Incubated with Increasing Concentrations of Rat Spinal Cord Membrane Fragments

Values, obtained after 3 days in culture as described in the Materials and Methods Section 2.2.4., are the means from 3 separate determinations using lymphocytes from different MND individuals and are expressed as the difference (in cpm or μg/ml) between test sample and that incubated in the absence of mitogen.



Rat spinal cord membrane protein fragments

3.1.8. Stimulation by Rat Spinal Cord Membranes Fragments of Lymphocytes from patients with Motor Neurone Disease

Lymphocytes from 14 MND patients (11 males, 3 females; age range 44-80 yrs : mean 62.4 yr), and 9 normal healthy controls (7 males, 2 females; age range 35-65 yrs : mean 55.3 yr) were cultured for 3 days in the presence of spinal cord membrane fragments at a sub-optimal concentration (50µg/ml). It was found that the glucose consumption assay gave extremely variable results in these experiments and no reliable data could be obtained. All the other assays gave consistent results and are shown in Fig. 3.7.

Lymphocytes from normal age-matched controls were non-reactive towards the membrane samples. However, even though MND lymphocytes responses were very variable, some patients showed stimulation greater than unity. For assays based upon the incorporation of radiolabelled ligand, a stimulation index greater than 3 has been suggested to be significant (Urbaniak et al, 1978) and by this criterion 4 out of 14 MND patients showed significant stimulation in all such assays.

3.1.9. Stimulation By Con A and PHA of Lymphocytes From Multiple Sclerosis Patients

Peripheral blood lymphocytes from 10 Multiple Sclerosis (MS)

patients (age range 18-41 yrs; mean 31.5 yr +_ S.D. 8 yr) and from 10 age-matched, healthy controls (age range 22-42 yrs; mean 31.8 yr +_ S.D. 7 yr) were stimulated with Con A and PHA for 3 days as described in Section 2.2.3.

The results are shown in Tables 3.3 (a) and (b) and Fig. 3.8 (a) and (b). In response to Con A, the incorporation of radiolabelled leucine and thymidine, and glucose consumption were all significantly decreased in MS patients compared to normals. Whereas, with PHA, all the above assays, and also the lactate release assay were significantly decreased compared to the normal healthy controls.

FIGURE 3.7 Stimulation by Rat Spinal Cord Membrane Fragments of lymphocytes from patients with Motor Neurone Disease

Values, obtained as described in the Results Section 3.1.8., are the means from triplicate assays of lymphocytes from each individual donor and are expressed either as the Stimulation Index (ratio of cpm from test sample to that from sample incubated in absence of spinal cord membranes) or as the difference between lactate released from test sample and that from the non-stimulated control.

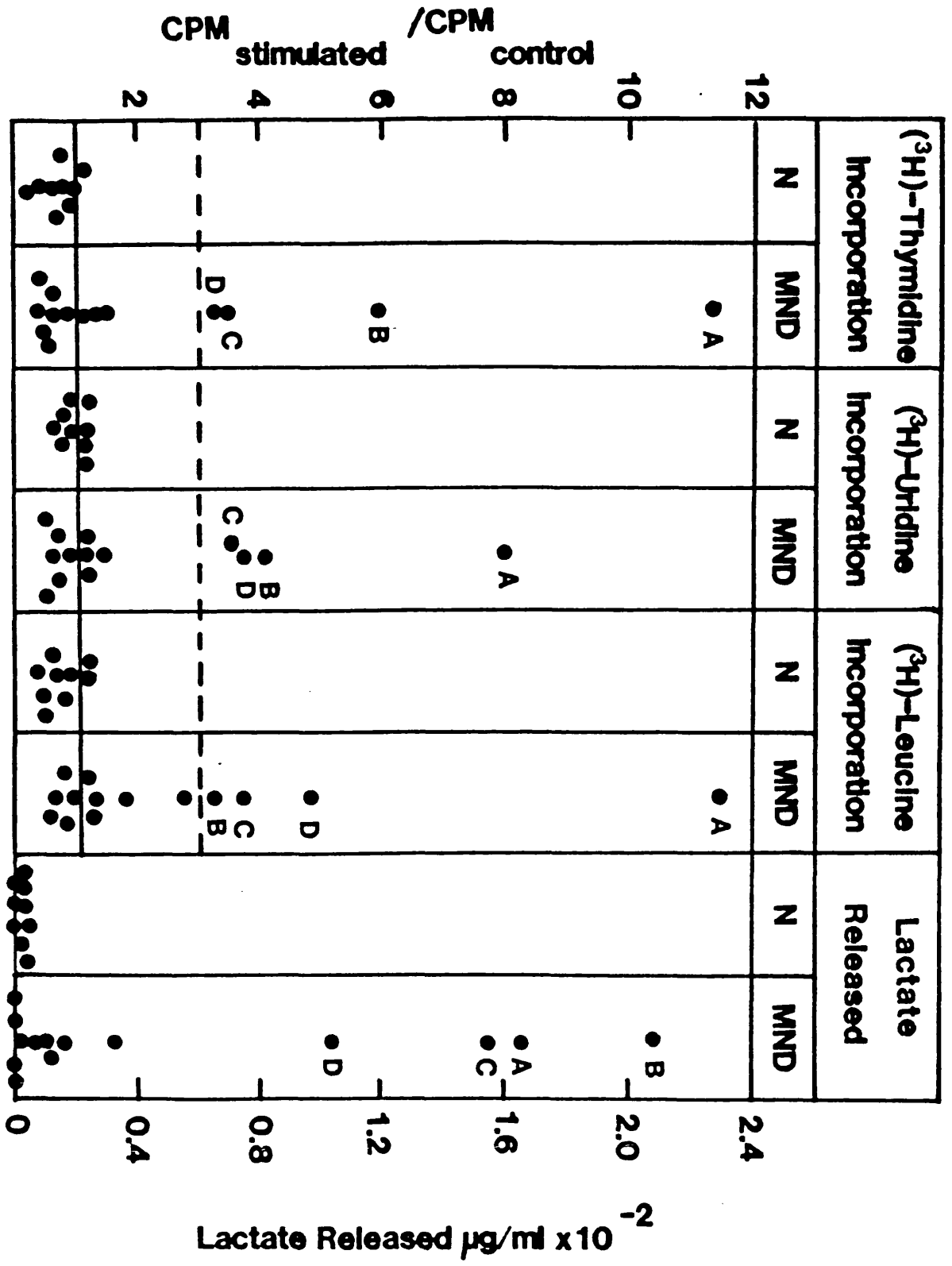

Stimulation Index
CPM stimulated /CPM control
2
4
6
8
10
12
(³H)-Thymidine Incorporation
(³H)-Uridine Incorporation
(³H)-Leucine Incorporation
Lactate Released
N
MND
A
B
C
D
0
0.4
0.8
1.2
1.6
2.0
2.4
Lactate Released µg/ml x10 -2

TABLE 3.3 (a) The Stimulation by Concanavalin A of Lymphocytes from Multiple Sclerosis

	NORMAL CONTROLS (10) MEAN = 31.8 yr +_ S.D. 7 yr	MULTIPLE SCLEROSIS (10) MEAN = 31.5 yr +_ S.D. 8 yr	SIGNIFICANCE P= 0.01
^{3}[H]-Thy Incorpor Log cpm	4.88+_0.17	4.19+_ 0.28	+
^{3}[H]-Urid Incorpor Log cpm	3.68+_0.35	3.82+_ 0.37	-
^{3}[H]-Leu Incorpor Log cpm	4.39+_0.21	3.66+_0.14	+
Lactate Release µg/ml	930+_218	713+_128	-
Glucose Consumed µg/ml	997+_187	367+_166	+

TABLE 3.3 (b) The Stimulation by Phytohaemagglutinin of Lymphocytes from Multiple Sclerosis Patients

	NORMAL CONTROLS (10) MEAN =31.8 yr +_S.D. 7 yr	MULTIPLE SCLEROSIS MEAN =31.5 yr +_ 8 yr	SIGNIFICANCE
^{3}[H]-Thy Incorpor Log cpm	4.85+_0.21	4.25+_0.25	+
^{3}[H]-Urid: Incorpor Log cpm	3.84+_0.27	3.87+_0.41	-
^{3}[H]-Leu Incorpor Log cpm	4.81+_0.12	4.32+_0.07	+
Lactate Released µg/ml	1281+_125	801+_198	+
Glucose Consumed µg/ml	1045+_224	482+_223	+

FIGURE 3.8 (a) The Stimulation by Con A of Lymphocytes from Multiple Sclerosis Patients and from Normal Healthy Controls

Values, obtained as described in the Results Section 3.1.9., are the means from triplicate assays of lymphocytes from each individual donor and are expressed as the difference (in cpm or μg/ml) between test sample and that incubated in the absence of mitogen.

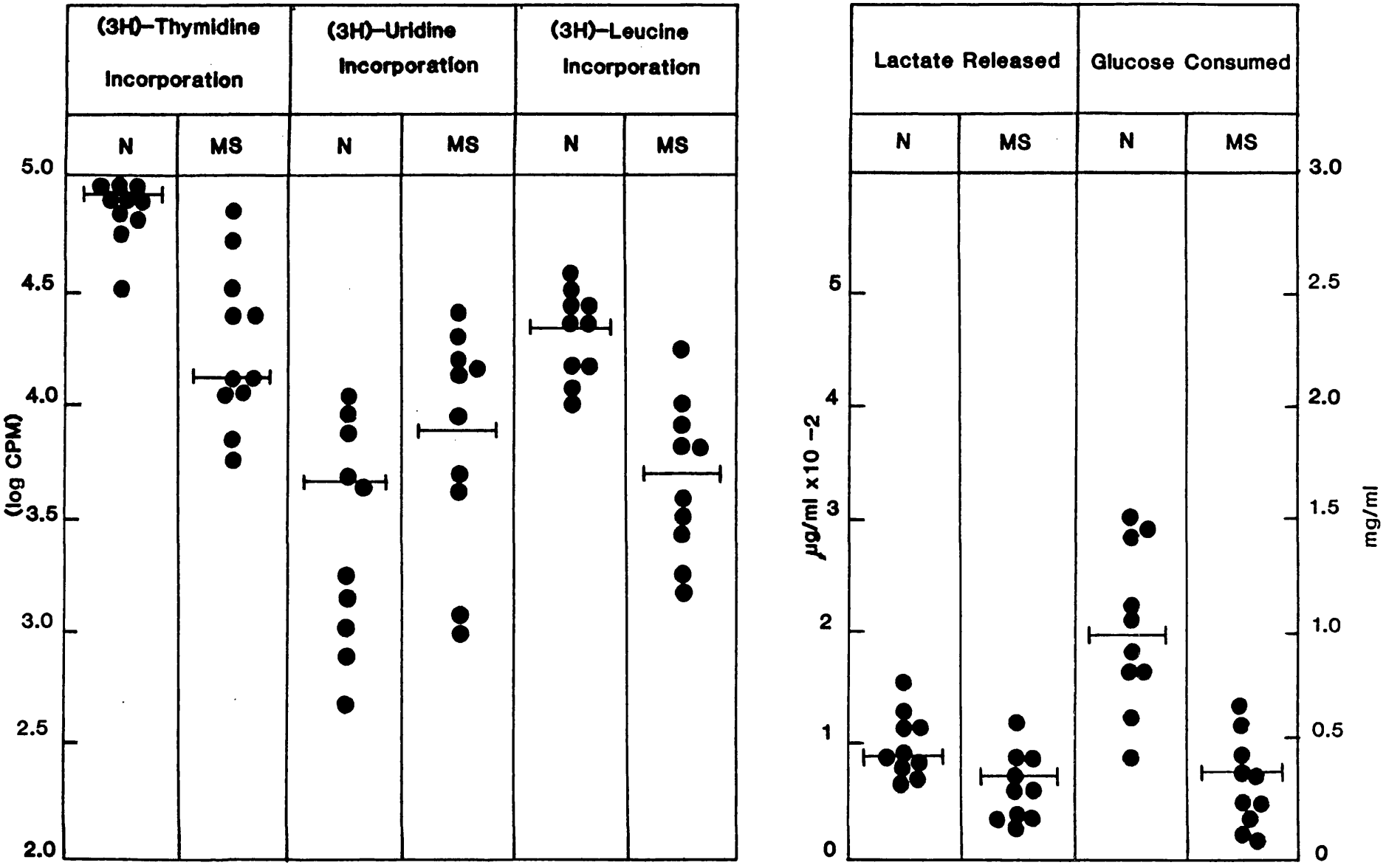
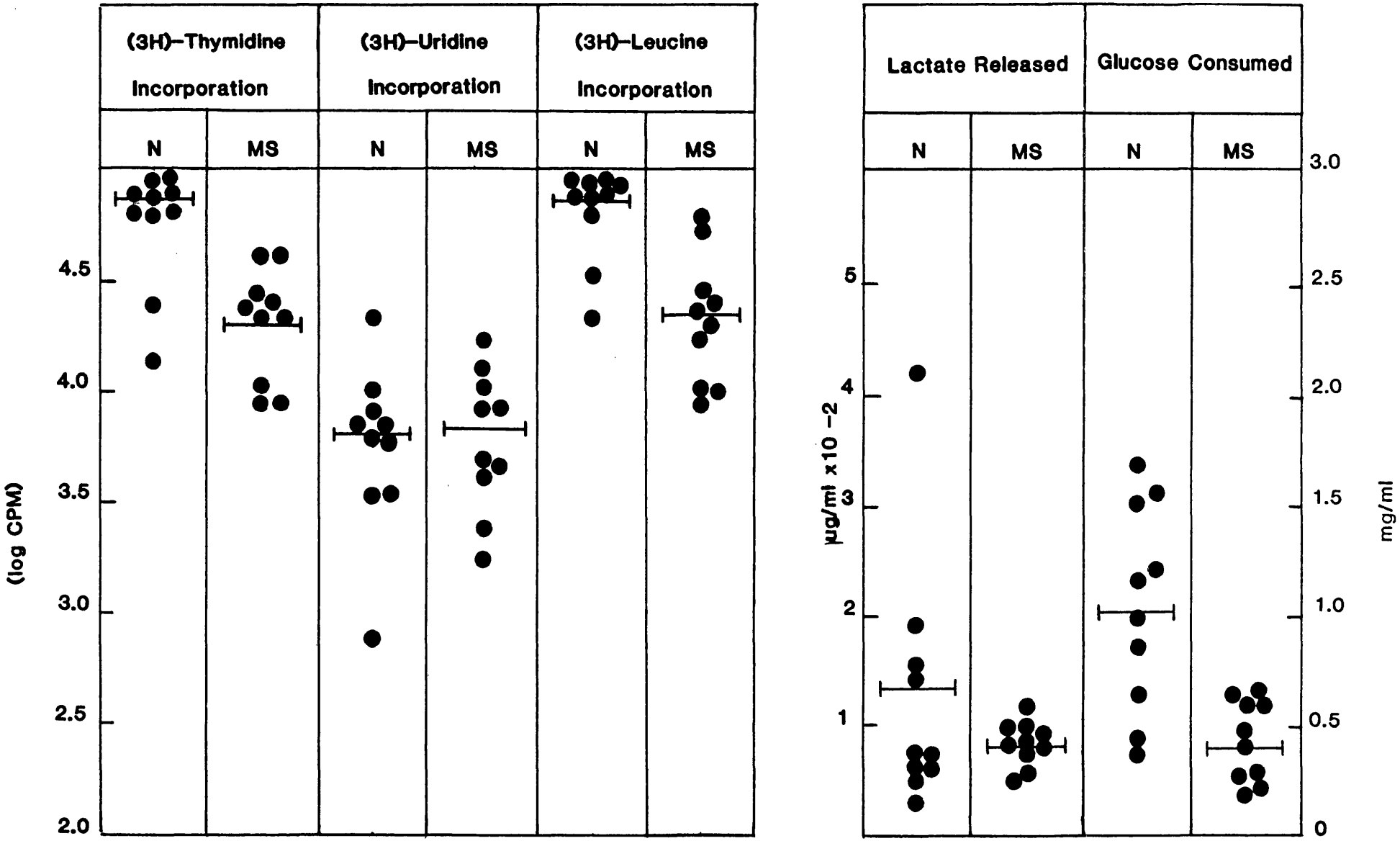

(3H)–Thymidine Incorporation
(3H)–Uridine Incorporation
(3H)–Leucine Incorporation
Lactate Released
Glucose Consumed
N
MS
N
MS
N
MS
N
MS
5.0
4.5
4.0
3.5
3.0
2.5
2.0
(log CPM)
5
4
3
2
1
0
µg/ml x10 −2
3.0
2.5
2.0
1.5
1.0
0.5
0
mg/ml

FIGURE 3.8 (b) The Stimulation By PHA of Lymphocytes from Multiple Sclerosis Patients and Normal Healthy Controls

Values, obtained as described in the Results Section 3.1.9., are the means of triplicate assays of lymphocytes from each individual donor and are expressed as the difference (in cpm or μg/ml) between test sample and that incubated in the absence of mitogen.


(3H)–Thymidine Incorporation
(3H)–Uridine Incorporation
(3H)–Leucine Incorporation
N
MS
N
MS
N
MS
(log CPM)
4.5
4.0
3.5
3.0
2.5
2.0
Lactate Released
Glucose Consumed
N
MS
N
MS
µg/ml x10 −2
5
4
3
2
1
mg/ml
3.0
2.5
2.0
1.5
1.0
0.5
0

3.1.10. Stimulation of Normal Lymphocytes by Pokeweed Mitogen : Optimisation of Conditions

The investigation of Pokeweed mitogen was undertaken because PWM is a polyclonal B cell activator and so enables the number of B-cells to be increased. The raising of human monoclonal antibodies was to be performed by fusing the mouse myeloma cells X63-Ag8.653 to human peripheral blood lymphocytes. The numbers of lymphocytes obtained from the peripheral blood are in the order of 30 million/20ml, of which only 30% are B-cells. Therefore the use of PWM increases the numbers of cells which could be potentially fused (Campbell, 1985).

3.1.10. (a) Time-Response Study

Peripheral blood lymphocytes from 5 normal healthy individuals (age range 22-44 yr : mean 29 yr) were incubated with Pokeweed mitogen for periods of upto 8 days. Lymphocyte proliferation was assayed by all five assays previously described.

The results, shown in Figure 3.9 show maximum stimulation after 72 h for all five assays.

3.1.10. (b) Dose-Response Study

Lymphocytes from five healthy controls (age range 23-28 yr : mean 25 +_ S.D. 2 yr) were stimulated with increasing concentrations of Pokeweed mitogen (0-100µg/ml) for 72 h in a humidified

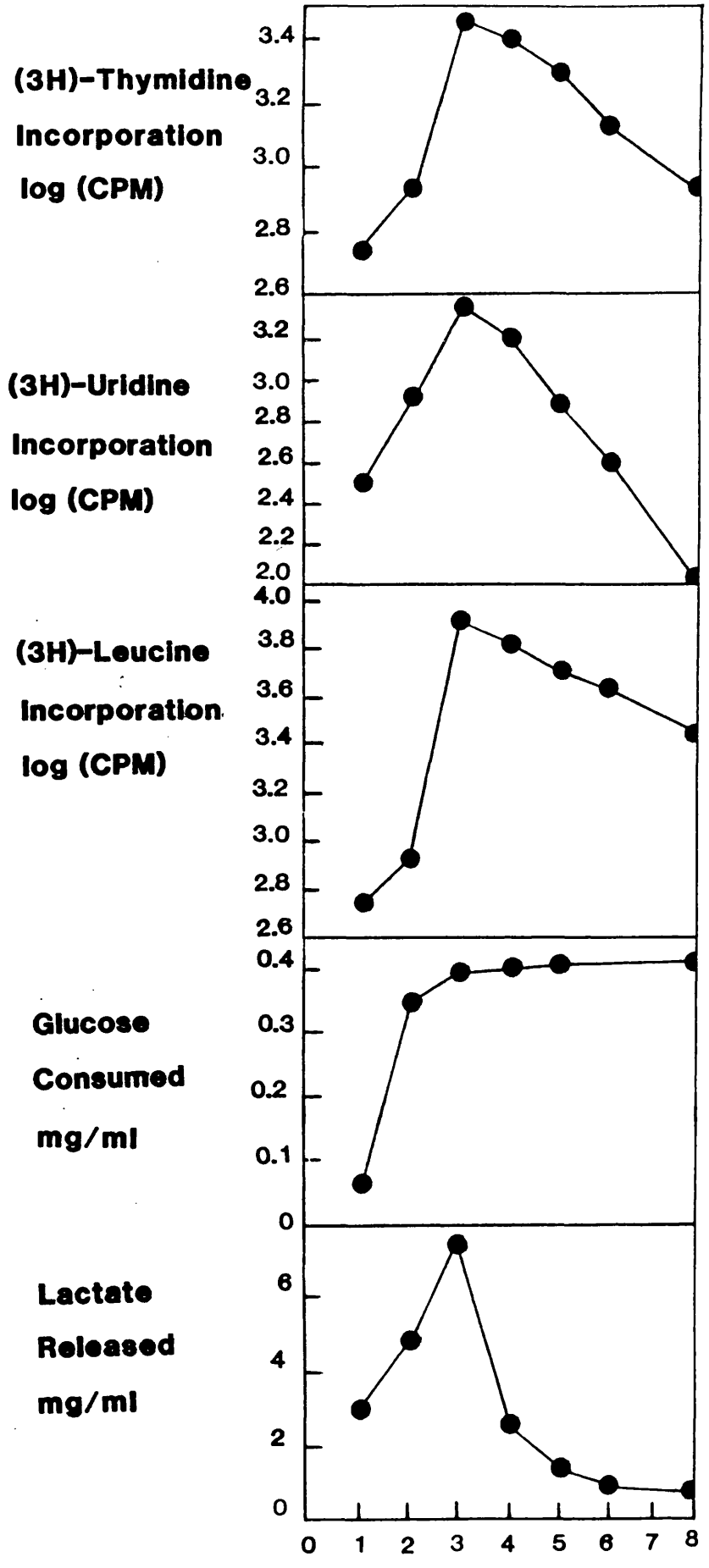


FIGURE 3.9 The Time–Response Study for PWM

atmosphere of 5% CO_2 in air at 37°C.

Lymphocyte stimulation was assayed by all five methods previously described and the results are shown in Figure 3.10. The concentration of 20 µg/ml gave maximal proliferation in all cases.

3.1.11. Stimulation of Normal Lymphocytes by Phytohaemagglutinin, Concanavalin A and Pokeweed Mitogen in Serum-Free Medium

The use of serum-free medium was investigated because the serum (fetal calf serum) used to supplement the lymphocyte culture medium is very expensive and it would be convenient to remove this.

Lymphocytes from 5 normal healthy individuals (age range 23-28 yr : mean 25 +_ S.D. 2.9 yr) were resuspended in two different media. One medium was the lymphocyte culture medium containing 10% (v/v) fetal calf serum (FCS), and the second was medium supplemented with insulin 0.2U/ml; human transferrin 5 µg/ml; progesterone 20 nM; putresceine 100 µM; sodium selenite 30 nM; hydrocortisone 0.5 µM; biotin 1 µg/ml; thyroxine 3 µg/ml and bovine serum albumin 1 mg/ml but without any FCS. Each control sample was divided into two aliquiots so that half was in the Serum-Supplemented Medium (SSM), and the other half in Serum-Free Medium (SFM). The samples were then stimulated in the usual way with PHA, Con A, and PWM for 3 days and the lymphocyte

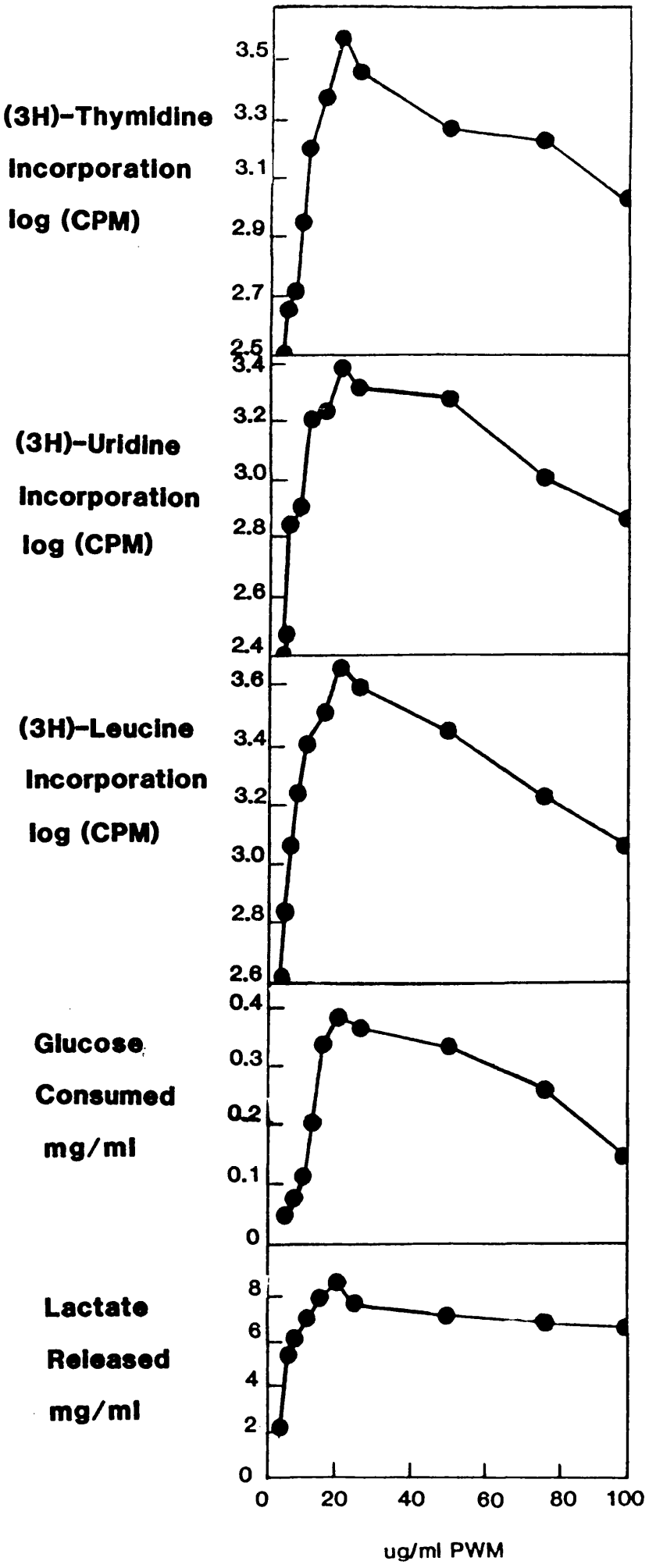


FIGURE 3.10 The Dose–Response Study for PWM

proliferation was measured by all five previously described assays.

The results are shown in Table 3.4. In SFM stimulation with PHA and Con A was virtually absent but the stimulation indices obtained with PWM are comparable with those shown in SSM.

3.1.12 Stimulation of Motor Neurone Diseased Lymphocytes by Direct Culture in the Presence of Foetal Rat Spinal Cord Neurones

An initial study was carried out to investigate the possibility that the peripheral blood lymphocytes from MND patients could be sensitized to spinal cords cells.

Trays (24 well) of 15 day old rat spinal cord cells cultured as described in Section 2.2.8. (a) were treated with mitomycin C for 1 h. Control preparations included wells with spinal cord cells but no lymphocytes and vice versa. Lymphocytes from MND patients were cultured in the spinal cord cell wells (0.1×10^6 /ml - 1×10^6 /ml) in a humidified atmosphere containing 10% CO_2 at 37 °C for 3 days. Lymphocyte proliferation was assayed by the incorporation of radiolabbelled thymidine, uridine and leucine.

The results, shown in Table 3.5, indicate that cultured rat spinal cord neurones cause stimulation of MND lymphocytes but not of normal controls.

TABLE 3.4 The Stimulation of Normal Lymphocytes by Con A, PHA, and Pokeweed Mitogen in Serum-Supplemented and Serum-Free Medium

	Concanavalin A		PHA		Pokeweed Mitogen	
	SFM	SSM	SFM	SSM	SFM	SSM
^{3}[H]-Thy Log CPM	0.77 +_0.12	3.7 +_0.22	1.31 +_0.12	3.78 +_0.14	3.30 +_0.13	3.73 +_0.15
^{3}[H]-Uri Log CPM	0.47 +_0.13	3.32 +_0.24	0.99 +_0.21	3.49 +_0.14	3.26 +_0.23	3.32 +_0.14
^{3}[H]-Leu Log CPM	1.09 +_0.11	4.28 +_0.23	1.54 +_0.19	4.64 +_0.24	3.25 +_0.21	3.38 +_0.15
Lactate Rel ug/ml	228 +_117	826 +_129	250 +_105	1454 +_138	710 +_213	887 +_156
Glucose Cons: ug/ml	32 +_11	1135 +_121	387 +_125	1245 +_135	1172 +_154	1124 +_149

SFM = Serum-Free Medium

SSM = Serum-Supplemented Medium

+_ Standard Deviation

Number of Experiments = 5

LYMPHOCYTE NUMBER	^{3}[H]-Thymidine INCORPORATION Log CPM	^{3}[H]-Uridine INCORPORATION Log CPM	^{3}[H]-Leucine INCORPORATION Log CPM
100,000			
MND	NS	NS	3.3+_ 0.2
NORMAL	NS	NS	NS
500,000			
MND	2.5+_0.21	2.7+_0.26	3.4+_0.3
NORMAL	NS	NS	NS
1,000,000			
MND	3.5+_0.30	3.81+_0.21	3.7+_0.12
NORMAL	0.81+_0.11	0.56+_0.32	0.39+_0.43

NS= no stimulation

Number of experiments = 3

Log CPM = (CPM of stimulated cells/ CPM of control cells)

Control cells

TABLE 3.5

Incorporation of Radiolabelled Isotopes into MND and Normal Lymphocytes Cultured with 15 Day Old Cultured Rat Spinal Cord Cells

SECTION 3.2 : RAT SPINAL CORD CELL CULTURE STUDIES

3.2.1. Cell Culture Conditions

Digestion of spinal cords with trypsin and DNAse gave 3.5-4.5 x 10^6 cells/ foetal spinal cord. The viability was > 90% with very good reproducibility.

When the cells were dissociated and plated out immediately in SFM, they failed to attach to the dishes and the few cells that did bind died after a few days. Cells which were kept in serum supplemented medium for the first three days showed attachment of the cells : serum free medium could then be used for subsequent growth (Digby et al, 1985).

3.2.2 Histochemical staining

Figures 3.11 (a)-(f) show cultures at various stages of maturity stained with Dalofeld's haematoxylin. Day 1 (Figure 3.11 (a))shows that, after 24 h, the majority of cells attach to the dishes. At this stage, it is possible to see flat cells on top of which are small oval neurone-like cells with some processes. By Day 3 (figure 3.11 (c)) the size and number of the processes have increased. The neurone-like cells have migrated to form clusters. However the flat cells also grow rapidly and can hinder the growth of the neurone-like cells (figure 3.11 (f)). This problem can be reduced by the addition of fluorodeoxyuridine and uridine

from day 6 to day 8. By day 12 (figure 3.11 (d)) the clusters have acheived maximum size and can be maintained for a further 10-12 days. Figure 3.11 (e) shows that some of the clusters are joined by processes which can become very thick.

3.2.4. Immunocytochemical Identification of the Cells

Mouse monoclonal antibodies were used in the identification of the cultured cells. A mouse monoclonal antineurofilament antibody confirms the neuronal identity of the clustered cells and their processes. This is shown in Figure 3.12. In no case were the underlying cells shown to be labelled with the anti-neurofilament antibody.

Tetanus toxin is a specific cell surface marker for neurones in culture (Mirskey et al, 1978). Figure 3.13 shows the labelling of the surface of the neurone-like clusters and their processes.

FIGURES 3.11 (a)-(f) Rat Spinal Cord Cells in Culture

Figure 3.11 (a) Photomicrographs of 1 day old rat spinal cord cells cultures stained with haematoxylin-eosin (a) low power (bar = 10μm) (b) high power (bar = 40μm)

Figure 3.11 (b) Photomicrographs of 2 day old rat spinal cord cells cultures stained with haematoxylin-eosin (a) low power (bar =10μm) (b) high power (bar = 40μm)

Figure 3.11 (c) Photomicrographs of 3 day old rat spinal cord cell cultures stained with haematoxylin-eosin (a) low power (bar = 10μm) (b) high power (bar = 40μm)

Figure 3.11 (d) Photomicrographs of 12 day old rat spinal cord cell cultures stained with haematoxylin-eosin (a) low power (bar= 10μm) (b) high power (bar = 40μm)

Figure 3.11 (e) Photomicrographs of 16 day old rat spinal cord cell cultures stained with haematoxylin-eosin (a) low power (bar = 10μm) (b) high power (bar = 40μm)

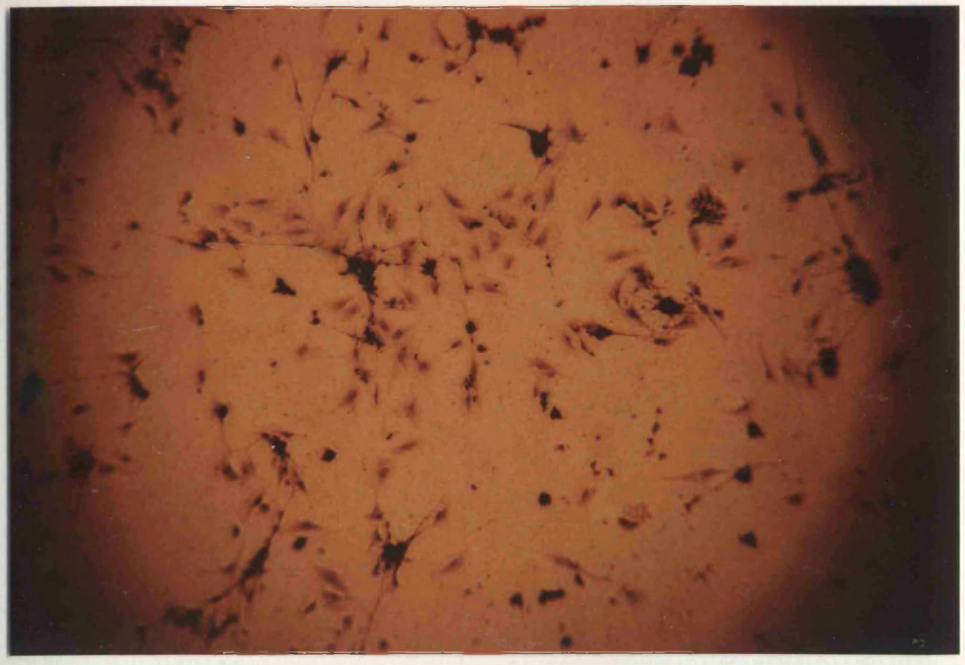
Figure 3.11 (f) Photomicrograph of a 3 day old rat spinal cord cell culture showing a variety of other cells low power (bar = 10μm)

(a)

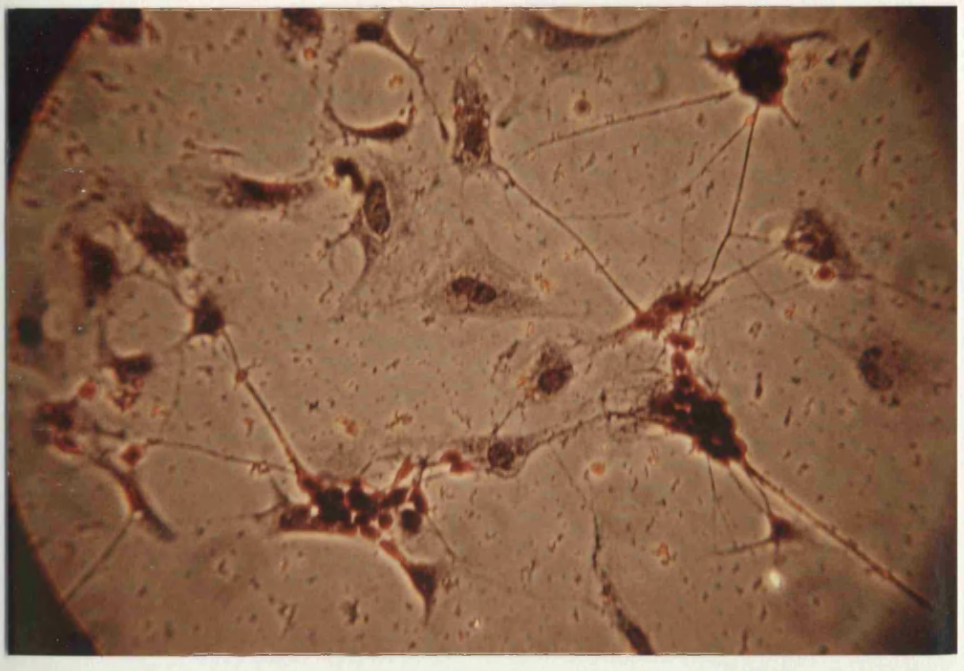
(a)

(b)

(b)

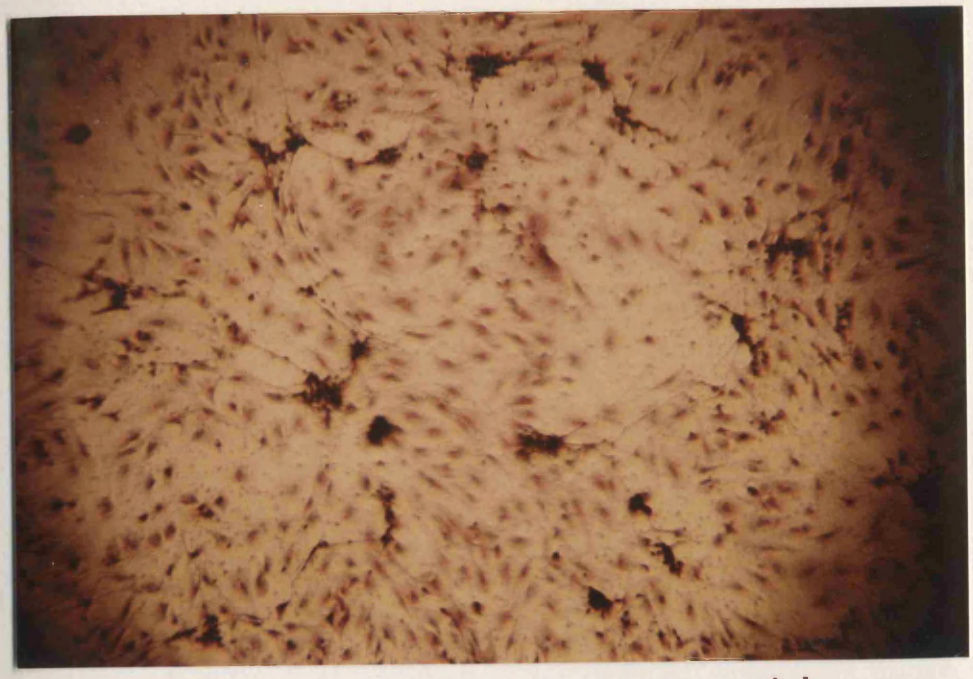


(a) ⊢⊣

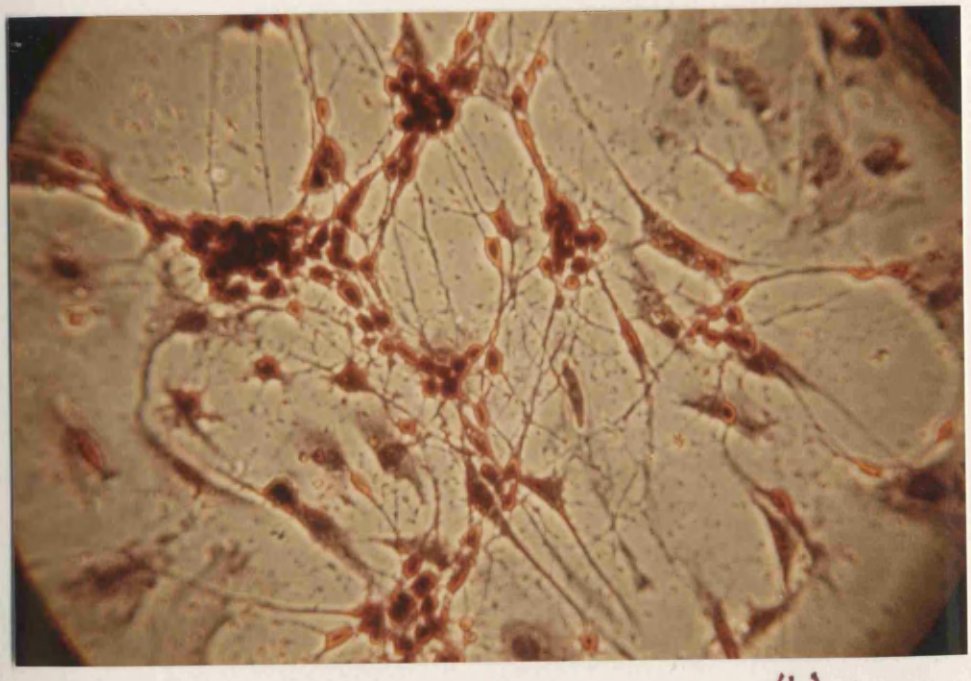


(b) ⊢⊣

(c)



(a) ⟝



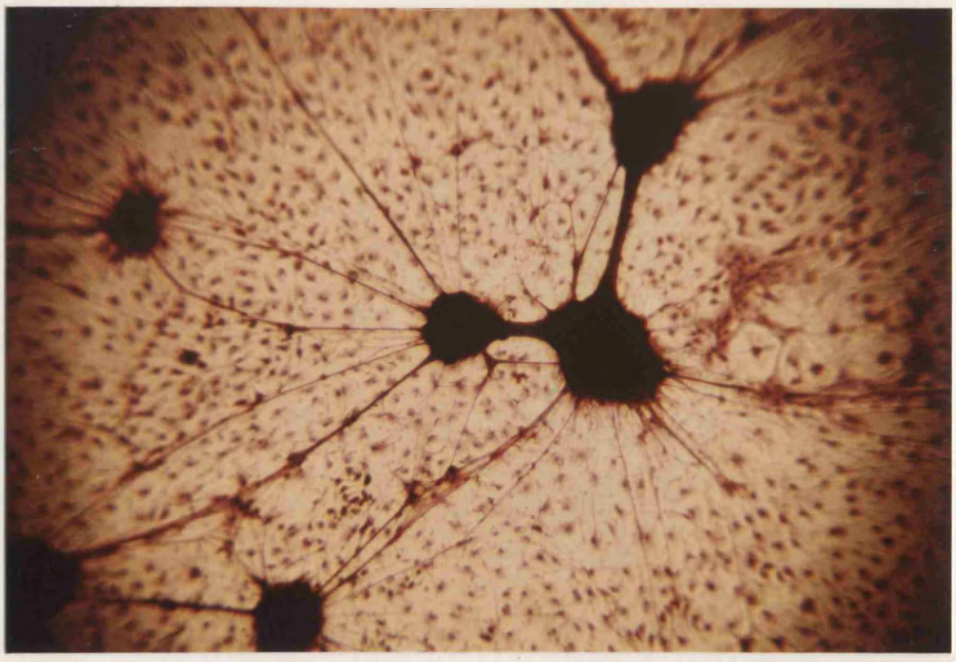
(b) ⟝

(d)

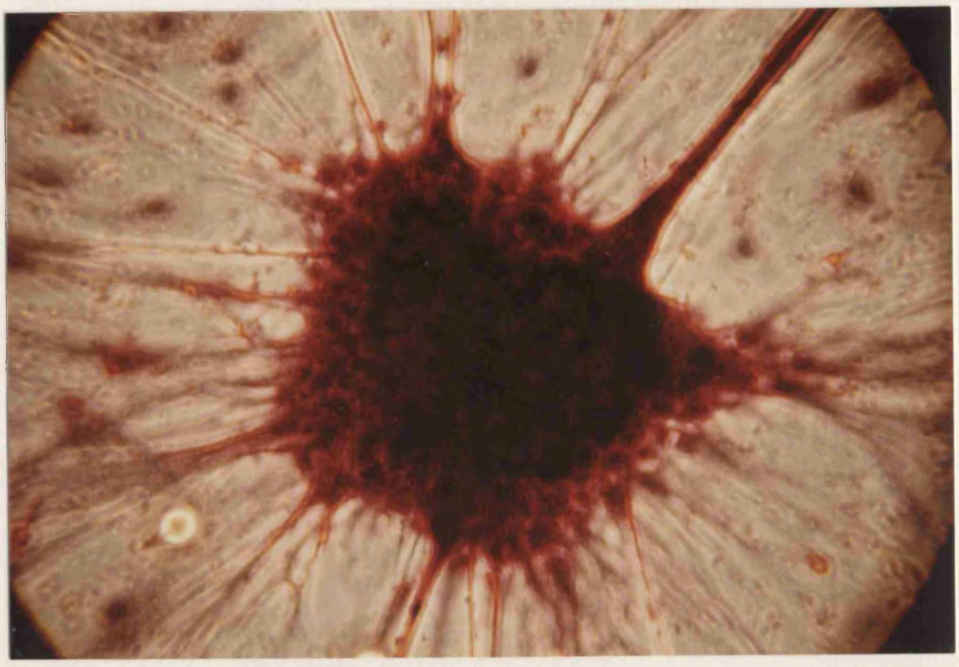
(a)

(b)

(e)



(a)



(b)

(f).

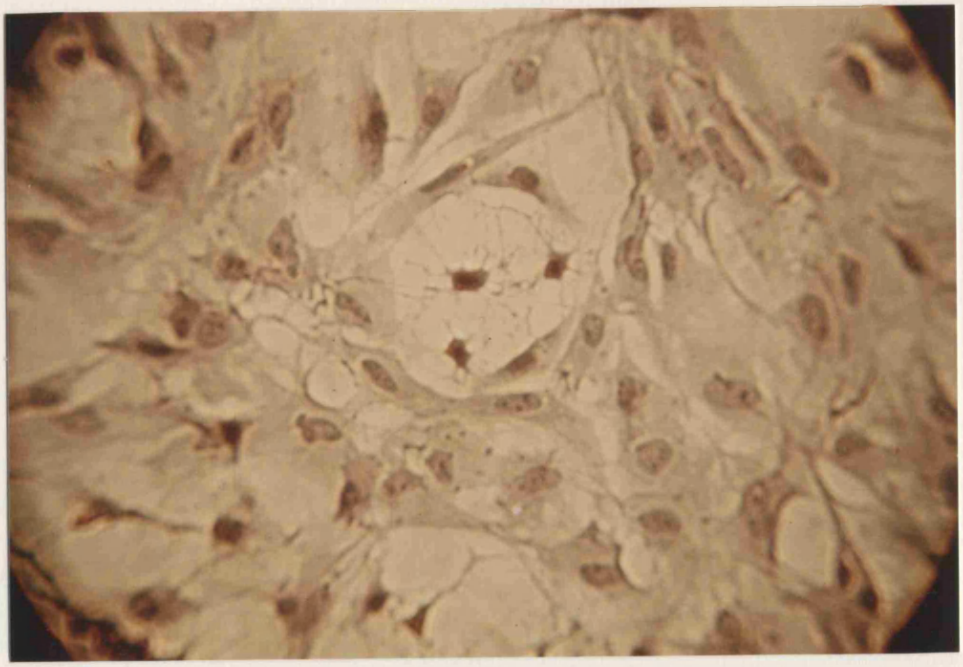
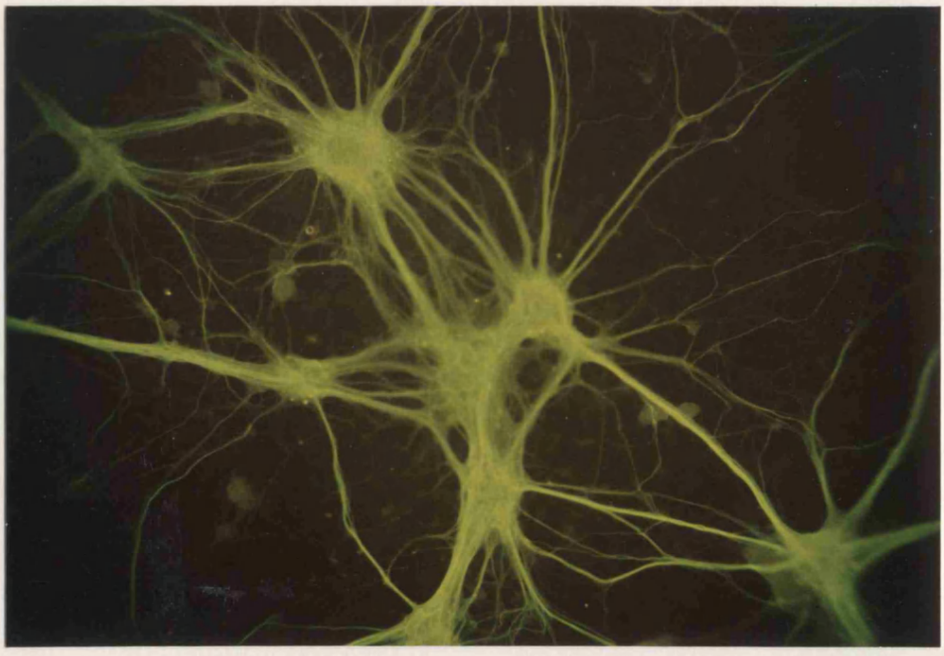


FIGURE 3.12

Rat spinal cord cells cultured for 12 days were fixed with acid alcohol and labeled with mouse antineurofilament antibody followed by fluorescein-labeled rabbit anti-mouse immunoglobulin and viewed with fluorescence optics. (a) low power (Bar =10μm) (b) high power (bar = 40μm)



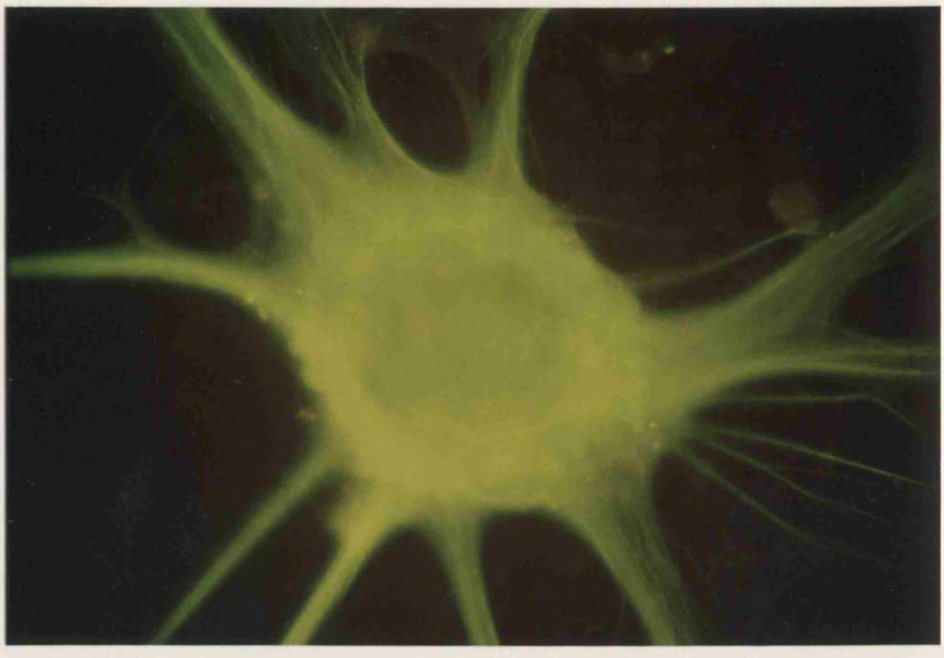
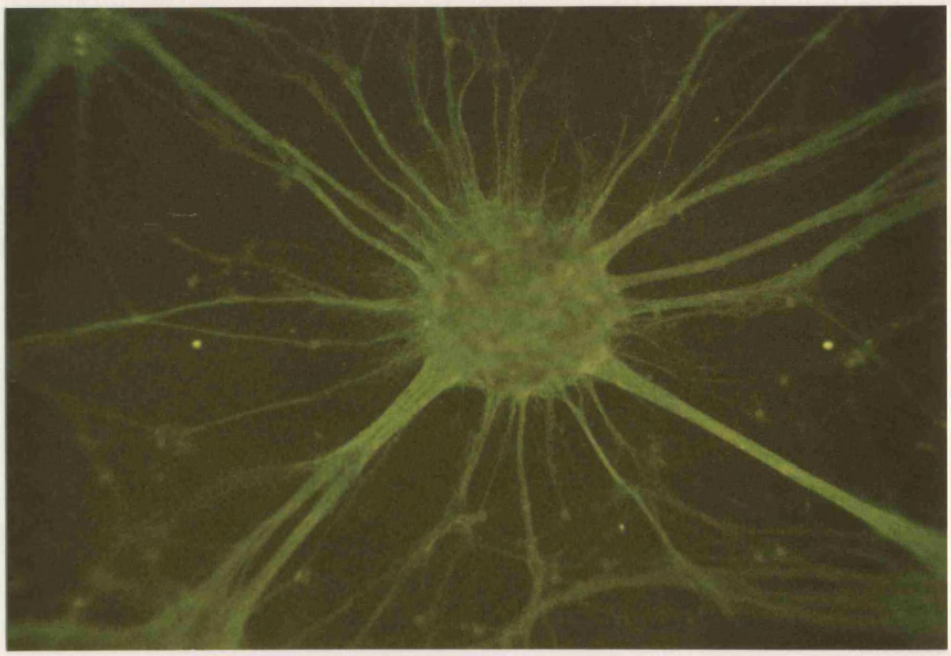


FIGURE 3.13

Rat spinal cord cells cultured for 12 days and labeled with tetanus toxin followed by mouse anti-tetanus toxin and finally by fluorescein-labelled rabbit anti-mouse immunoglobulin viewed under fluorescence optics high power (bar = 40µm)



3.3 HUMAN MONOCLONAL ANTIBODY PRODUCTION

3.3.1. Detection of Immunoglobulin Production

3.3.1. (a) Immunodot Assay

The assay was performed as described in Section 2.2.12. (a) and used to detect whether colonies were actually secreting immunoglobulin. It was a very sensitive system detecting levels at 500 ng/ml. However, when the class of immunoglobulin being secreted was investigated, the culture supernatants often gave positive results for more than one class of immunoglobulin. This could have been due to the assay system being too sensitive and detecting left over immunoglobulin when there was more than one colony in a particular well.

The assay was not a very quantitative procedure as it was extremely difficult to distinguish between the intensity of colour of one "spot" from another. However a first screening procedure it was quick and easy to perform.

3.3.1. (b) CELIA

For the CELIA, standard curves for IgG, IgM, and IgA were constructed. This enabled the class and quantity of the immunoglobulin to be determined from each colony.

The procedure is complex and takes a long time to perform, especially if there are many culture

FIGURE 3.14 The Standard Curves of IgG, IgM, and IgA using CELIA

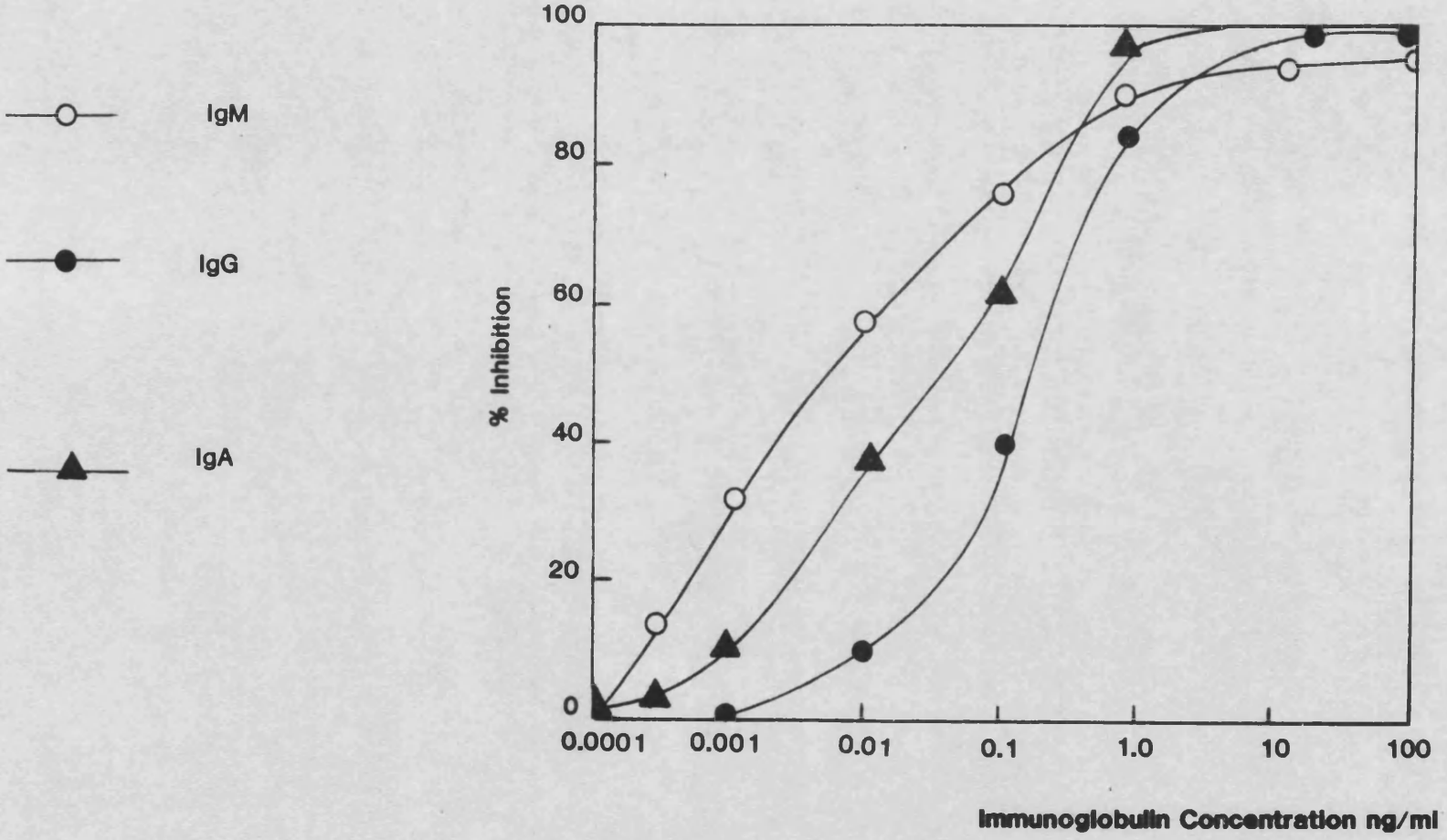

IgM
IgG
IgA
100
80
60
40
20
0
% Inhibition
0.0001
0.001
0.01
0.1
1.0
10
100
Immunoglobulin Concentration ng/ml

FIGURE 3.15 Double antibody Sandwich Enzyme Linked Immunoabsorbent Assay with Alkaline Phosphatase as Substrate

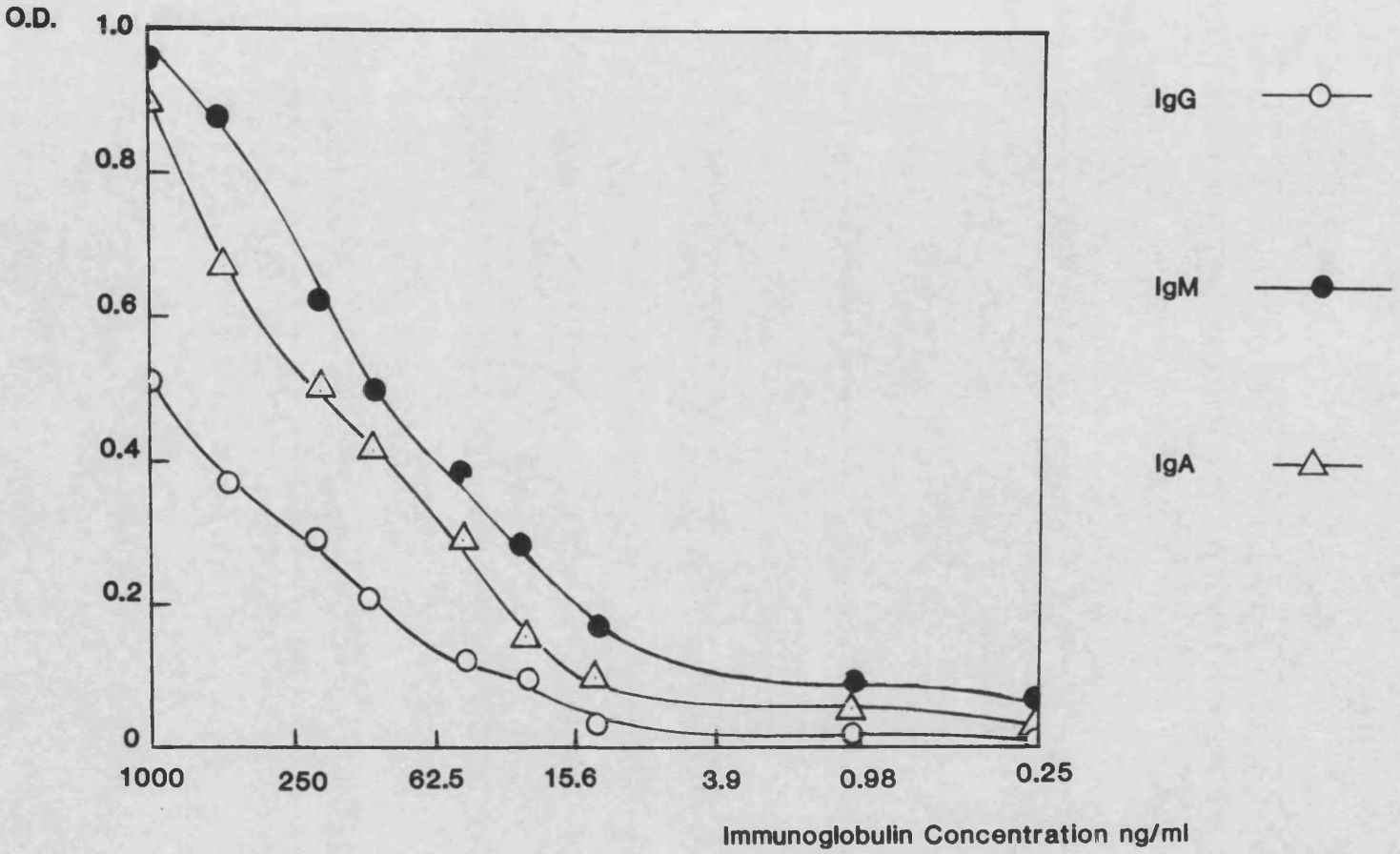
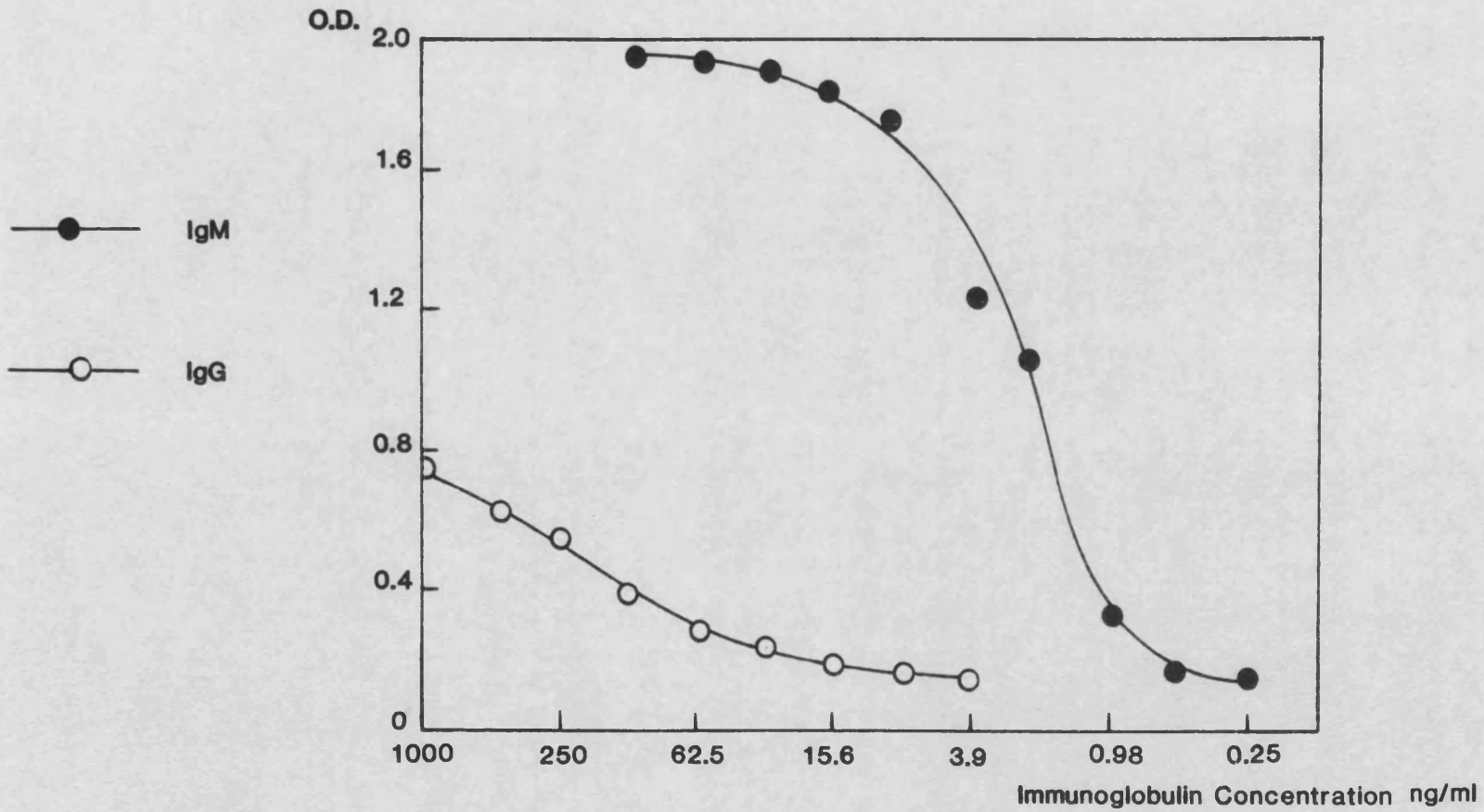

O.D.
1.0
0.8
0.6
0.4
0.2
0
1000
250
62.5
15.6
3.9
0.98
0.25
Immunoglobulin Concentration ng/ml
IgG
IgM
IgA

FIGURE 3.16 Double Antibody Sandwich Enzyme Linked Immunoabsorbent Assay with Horse Radish Peroxidase as Substrate


O.D.
2.0
1.6
1.2
0.8
0.4
0
IgM
IgG
1000
250
62.5
15.6
3.9
0.98
0.25
Immunoglobulin Concentration ng/ml

supernatants to be assayed. The results obtained by this procedure were very variable from day to day and not at all easy to optimise. However the procedure decided upon is described in section 2.2.12.(b). The standard curves are shown in Figure 3.14. A standard curve was run with each assay to account for variability.

3.3.1. (c) Double Antibody Sandwich ELISA

The assay was performed as described in Section 2.2.12.(c), and was developed because of the increase in sensitivity compared with the CELIA. Standard curves were established for IgG, IgM, and IgA and are shown in Figures 3.15 and 3.16. The coating solution was found to give the best results at 100 ng/ml Sheep Anti-Light chain antibodies (SAL) in sodium carbonate buffer 50 mM, pH 9.6. The standard curves were used to determine the amount and class of immunoglobulin secreted by the colonies.

This method was more convenient than the CELIA to use and gave consistently reliable results. The variation of the standard deviation was less than 2 % from day to day on the same sample culture supernatant. Two different enzyme conjugates were used, alkaline phosphatase and horse radish peroxidase. Horse radish peroxidase was found to be the more sensitive of the two as can be easily seen from the two sets of standard curves (Figs 3.15 and 3.16).

3.3.1. (d) ELISA with Rat Spinal Cord Membranes

Culture supernatants were tested on rat spinal cord membrane fractions (prepared as described in Section 2.2.8. (b)). A number of different coating antigen concentrations were tried to find the optimum concentration. Table 3.6 summarises the results.

COATING ANTIGEN n=5 (μg/ml)	OPTICAL 405 nm DENSITY
0.5	0.010
1.0	0.798
5.0	0.625
10.0	0.652

TABLE 3.6. Optimisation of coating antigen concentration with the culture supernatant from a positive clone obtained from a MND patient

Maximum reaction of antibody was with antigen coated at 1 μg/ml in 50 mM sodium carbonate buffer pH 9.6. These conditions were then used for any subsequent reactions.

This assay system was used to detect any anti-rat spinal cord membrane antibodies secreted by clones obtained after

peripheral blood lymphocytes from MND patients, MS patients, and normal healthy controls were either fused with a mouse myeloma cell line X63-Ag8.653 or transformed with Epstein-Barr virus. Positive results were those wells that gave 50% more colour than the control wells which contained culture supernatants from colonies formed from healthy normal individuals and reagent blanks. Colonies whose supernatant was 50% greater than control wells using the ELISA, tested twice were expanded in flasks and then recloned by limiting dilution at cell concentrations of 0.5, 5, and 50 cells/well. The cell concentration of 5 cells/well gave the best results; 0.5 cells/well yielded no colonies and 50 cells/well yielded 90% of wells with colonies. The supernatant from the expanded clones was stored and concentrated for future use. Samples of each positive clone obtained were frozen and stored in liquid nitrogen.

3.3.2. Human Monoclonal Antibody Production

Three separate methods were used for the production of human monoclonal antibodies. These were the fusion of peripheral blood lymphocytes (PBL) with a mouse myeloma cell line X63-Ag8.653 in the ratio 1:1. The second method was the prior stimulation of the PBL with Pokeweed mitogen at the optimal concentration (20 ug/ml) followed by fusion as described above. The final procedure used was the intial stimulation of the PBL with the Pokeweed mitogen and then the transformation of the cells with Epstein-Barr virus.

The growth and number of colonies obtained by each method will be discussed separately. When the colonies were visible by eye, screening was carried out with all the previously described detection assays.

3.3.2. (a) Fusion of PBL with X63-Ag8.653

The fusion of the mouse myeloma X63-Ag8.653 cell line with human peripheral blood lymphocytes results in slower growth, fewer clones and lower immunoglobulin levels secreted than is the case with mouse-mouse, rat-rat, or even human-human hybridomas. The clones began to appear after 3 weeks, but even after 6 weeks some further clones started to appear. The early assaying of the clones is essential in order to obtain the required hybrid as they are easily lost; mouse-human hybrids have been reported to be unstable and need recloning immediately. Fusions were performed with PBL from MND, MS and normal healthy controls. Previous results had indicated that lymphocytes from the MND patients could be stimulated by rat spinal cord membrane fragments suggesting that the lymphocytes are sensitized to these fragments. The results are summarised in Table 3.7.

The immunoglobulin production ranged from 7 ng/ml to 1 µg/ml with an average of 200 ng/ml. The wells that produced colonies were mainly IgG secretors with a few IgM secretors. From the 3 fusions performed on PBL from the MND patients a total of 20 wells were positive of which 50% were frozen and the remainder

were recloned by limiting dilution. After recloning, only 6 wells remained positive, these were expanded in flasks and some of each of the cells frozen whilst the remainder were kept in culture. The immunoglobulin production of the 6 wells was low averaging 200 ng/ml. However 4 started to lose their immunoglobulin production until, after a couple of months, in culture nothing could be detected. The 2 remaining producers were IgG secretors and remained stable.

The MS fusions produced 4 positive clones all of which were IgG secretors (300-750 ng/ml). Two of the clones were recloned whilst the other two were frozen. Upon recloning by limiting dilution a further 4 positive clones were also detected. These were IgG secretors (250-750 ng/ml) and were expanded in flasks so that some of the cells could be frozen while the remainder were kept in culture. Their immunoglobulin secretion was very stable.

The fused normal samples gave many colonies of which 8 were selected and 4 were subsequently frozen. The remaining 4 were recloned by limiting dilution and 4 colonies were chosen for their immunoglobulin production (around 0.8 µg/ml). Of these, 2 gave IgG; 1, IgM; and 1, IgA. These were kept in culture as controls, their immunoglobulin production was fairly stable.

TABLE 3.7

FUSED SAMPLE	NO. Of CELLS: FUSED (10^6)	% WELLS: GROWTH	+ WELLS: R.S.C.	+ WELLS: M.B.P.	%IgG	%IgM	RECLONED
MND 1	26		4	0	70	20	+
2	22	40-50%	6	0	78	16	+
3	18		8	0	85	12	+
MS 1	31	10	0	2	75	20	+
2	33	12	0	2	77	21	F
NORM.1	36	30	0	0	90	8	+
2	41	50	0	0	95	3	F

F = FROZEN IN LIQUID NITROGEN

+ = RECLONED BY LIMITING DILUTION

NORM. = NORMAL BLOOD SAMPLE

MND = MOTOR NEURONE DISEASE BLOOD SAMPLE

MS = MULTIPLE SCLEROSIS BLOOD SAMPLE

R.S.C. = RAT SPINAL CORD MEMBRANES

M.B.P. = MYELIN BASIC PROTEIN

3.3.2. (b) Pokeweed Mitogen Stimulation of PBL and then Fusion with a mouse myeloma cell Line

PBL from 2 MND patients, 2 MS patients and 2 normal healthy controls were first stimulated with optimal concentrations of PWM and then fused with X63-Ag8.653. The results were similar to the above except that after the stimulation for 5 days with PWM the numbers of lymphocytes increased, enabling more cells to be fused. This was very advantageous as the numbers of lymphocytes recovered from diseased patients' blood is often low.

The same percentage of wells yielded colonies, with a similar percentage of IgG and IgM secretors. The immunoglobulin production was still low with an average of 250 ng/ml. From the 18 MND clones positive for rat spinal cord membrane fragments, 9 were frozen and 9 recloned by limiting dilution. Of the 9 positives recloned only 10 wells were positive of which all stayed positive for at least a month. However, these were subsequently lost because of a fungal infection. The 6 positive MS clones were all recloned by limiting dilution and yielded 4 positives which were expanded. Cells from each clone were frozen and the rest were maintained in culture. The immunoglobulin levels stayed the same for 3 months. The normal fusions gave many colonies of which 4 (2 IgG and 2 IgM secretors) were also recloned and 4 colonies (2 IgG and 2 IgM secretors) were then maintained in culture as controls. The results are summarised in Table 3.8

TABLE 3.8

FUSED SAMPLE	NO. OF CELLS FUSED (10^6) B	A	%WELLS GROWTH	+WELLS R.S.C.	+WELLS M.B.P.	%IgG	%IgM	RECLONED
MND 1	19	23	40-50	10	0	86	11	+
2	21	24		8	0	81	13	F
MS 1	25	29	30-50	0	6	92	4	+
2	32	36		0	2	95	2	F
NORM.1	35	41	40-50	0	0	89	4	F
2	36	42		0	0	93	4	+

F = FROZEN

+ = RECLONED BY LIMITING DILUTION

R.S.C. = RAT SPINAL CORD MEMBRANE

M.B.P. = MYELIN BASIC PROTEIN

B = BEFORE STIMULATION WITH PWM

A = AFTER 5 DAY STIMULATION WITH PWM

3.3.2. (c) Epstein-Barr Transformation of Human PBL

The Epstein-Barr transformation of PBL enables the B-cells to proliferate and secrete immunoglobulin without having to fuse the cells with a myeloma cell line. The fusion of cells causes cell death if not performed correctly so this method only allows minimal handling of the cells. The EBV transforms the majority of B-cells in each blood sample. However, here the lymphocytes were separated and then initially stimulated with Pokeweed mitogen (20 ug/ml) for 5 days to increase the number of B-cells. They were then transformed with EBV for 1 h and finally plated out, as for any other fusion.

The cell growth was quick, the plates being covered with colonies after only 2 weeks. Samples were 4 MND, 1 MS and 1 normal. The total number of positive wells was 21, of which half were IgG secretors, and the other half IgM secretors. The level of immunoglobulin production was still found to be low, averaging 0.50 ug/ml. Seven of the positives were recloned by limiting dilution. These were chosen because of their greater immunoglobulin production. The remainder were frozen. From these 7 came 14 positives of which half were producing IgG and half producing IgM. These were expanded in flasks and some of each frozen, the rest were kept in culture and their levels of immunoglobulin stayed stable (0.75 ug/ml).

The MS transformation gave only 2 positives and they were

IgM secretors having values of 0.50 µg/ml, and 0.70 µg/ml. These were recloned by limiting dilution and 4 positives were selected 2 of which were frozen and the other 2 were kept in cuture. They remained stable for 4 months.

The transformation of normal cells gave many colonies none of which was positive in the MS or MND ELISA. However, 2 strong secretors of IgG (0.45 µg/ml) and IgM (0.30 µg/ml) were selected and maintained in culture. These, even without recloning, still continued to secrete immunoglobulin. The results are summarised in Table 3.9

3.3.3 Immunocytochemical Identification

All the positive clones described above and culture supernatants from normal clones were tested on fixed and unfixed cultures of rat spinal cord neurones (15 day) by immunofluorescence.

The MS supernatants did not give any labelling with the antibody. This could be expected as the cultures may not be myelinated. The normal supernatants gave the same labelling as the reagent blanks.

The MND supernatants showed no noticable difference from the background controls. These results could be explained if the affinities of the antibodies in the supernatants are low or if, even after concentrating by salt fractionation, the amount of immunoglobulin present was still too low to be detectable.

TABLE 3.9

FUSED SAMPLE	NO. OF CELLS: 6 FUSED(10)	% WELLS: GROWTH	+ WELLS: R.S.C	+ WELLS: M.B.P.	%IgG	%IgM	RECLONED
MND 1	20		5	0	55	40	+
2	23		4	0	56	42	+
3	26	90-70	6	0	54	41	+
4	27		6	0	55	42	+
MS 1	30	90-70	0	6	56	42	+
2	32		0	7	53	45	F
NORM 1	35	75-60	0	0	55	41	+

F = FROZEN

+ = RECLONED BY LIMITING DILUTION

R.S.C. = RAT SPINAL CORD MEMBRANE

M.B.P. = MYELIN BASIC PROTEIN

CHAPTER 4 DISCUSSION

4.1. Stimulation of Lymphocytes by Mitogens

Over the past two decades there has been a significant increase in studies relating to the function of the immune system. The continued popularity of research in this area reflects the awareness by the medical and scientific professions of the importance of immune function in the maintenance of disease-free homeostasis and technological advances in the field of immunological research.

Human lymphocyte transformation response to mitogen is one of the most widely used assays of cell-mediated immune function, and has proved to be an excellent immunodiagnostic tool. Lymphocytes are cells which can be stimulated to proliferate and undergo blastic transformation by a variety of biological and non-biological agents (see Introduction Section 4). This blastogenic responsiveness of peripheral blood lymphocytes has been used to gauge the immune response in a variety of diseases and is generally used to assess the responsiveness of groups of people rather than the repeated testing of one indivdual which tends to show large variations (Buckley, 1976; Oppenheim and Schecter, 1976). Cancer patients are kept under constant observation as their immune function ranges from normal or near normal to profound depression depending on the type and stage of the disease (Levy, 1978). Immunodeficiency states frequently show defective lymphocyte reactivity *in vitro* eg. in Di-George's

syndrome, T-cells are deficient but there are B-cells capable of making an antibody response (Roitt et al, 1985). Autoimmune diseases often are associated with abnormalities in cellular immunity with reduced lymphoproliferative reactions in vitro to T-cell mitogens as in Rheumatoid Arthritis (RA)(Yamana, 1978) and Multiple sclerosis (MS)(Tosato,1985). There are defective lymphocyte subpopulations in a variety of diseases (Bach and Bach, 1981; Nowinski et al, 1983). In a number of liver diseases the T-cell subsets are abnormal, in MS the subsets are also altered. In parasitic diseases evaluation of human T suppressor lymphocyte subpopulation may indicate how chronic the infection actually is e.g. in the case of leprosy which is a chronic bacterial disease depending upon cell mediated immunity entirely for protection. The role of T-cells is important and lymphocyte transformation using whole or sonicated mycobacterium leprae as antigen is used to assess the immune status of the individual (Mshana et al, 1982; Bloom, 1983).

The stimulation of B-cells in vitro with mitogens causes them to secrete immunoglobulin and provides valuble information about various aspects of B-cell function, e. g., antibody response repertoire.

The purpose of the present investigation was to study, under optimal conditions, lymphoproliferative responses, from diseased (largely MND) patients and normal controls, to a variety of mitogens.

The measurement of lymphocyte stimulation indices relies upon a variety of factors such as the dose of mitogens used, the incubation time with the mitogens, the way of expressing the stimulation indices and the overall assay systems selected.

In this study, to increase the chances of detecting abnormality, 5 assays were employed. Three of which relied on the measurement of radiolabelled ligand incorporation into macromolecules, namely, DNA, RNA, and protein. The other two methods measured glycolysis in stimulated cells. The methods used for the incorporation of radio-labelled precursors rely on pulse labelling for 18 h. Hence these methods only look at the events at the time of and during pulse labelling and do not measure changes taking place during the whole time of incubation with the mitogen under study. Therefore, glucose consumption and lactate released were also measured as they give an overall estimation of changes in energy (De Cock et al, 1980; Cordialifei et al, 1980).

Another reason for using five different assays was the fact that even with the strongest lymphocyte stimulants, the response of the cells was heterogeneous. With PHA, for instance, a high proportion of cells respond by increased RNA and protein synthesis but only about half the cells initiate DNA synthesis (Ling and Kay, 1975). Hence, thymidine uptake would not detect changes in those cells that do not replicate.

These 5 assays were used to investigate peripheral blood

lymphocytes from MND and MS patients compared to age-matched controls. As the abnormality in autoimmune disorders is often in T-cell control of the immune regulation, two mitogens Concanavalin A and Phytohaemagluttinin both of which are reported to be T-cell specific (Ramagnani, 1977) were utilised. PWM stimulation was also investigated.

Dose-response and time -response curves were determined for normal lymphocytes in order to establish the best conditions for measuring stimulation indices. With both mitogens Con A and PHA maximum response was obtained with 10 ug/ml. Most laboratories standardise assay conditions to produce maximum responses according to the idea that, under such conditions, maximum differences will be seen between patients and controls (Oppenheim et al, 1974). However, in this study sub-optimum doses were employed for subsequent work because sub-optimum mitogen concentrations give maximum differences between normal and impaired lymphocyte responses (Fitzgerald, 1971; Malave et al, 1975; Waller and Maclennan, 1977; Bernhard et al, 1980).

The time-response curves for all mitogens showed a maximum response by day 3 for [^{3}H]-thymidine and [^{3}H]-leucine uptake. While [^{3}H]-uridine uptake peaked after 48 h. Glucose consumption also reached a steady level after 3 days. Hence for the convenience of assaying day 3 was chosen as the incubation period for all subsequent assay. Others (Bernard et al, 1980) also found that this gives the greatest discrimination between

normal and diseased lymphocytes.

The responses of the 18 MND patients' peripheral blood lymphocytes (PBL) were found to be significantly depressed ($p < 0.01$) in response to Con A and PHA relative to the controls as measured by the [^{3}H]-thymidine, [^{3}H]-leucine incorporation and by the amount of glucose consumed. The [^{3}H]-uridine incorporation and the lactate release assays gave similar results to those of normal healthy controls.

Although the [^{3}H]-uridine and the lactate release assays failed to show a significantly depressed response, there is no reason to expect a correlation between uridine incorporation and that of thymidine or leucine nor between lactate release and the glucose uptake assays.

As mentioned earlier the mitogenic response of cells is very heterogeneous even with the strongest lymphocyte stimulants. The incorporation of uridine into RNA is greatly increased upon stimulation with mitogens and becomes prominent well before DNA synthesis. The time-response curves (Section Results 3.1.5.) show that the incorporation of [^{3}H]-uridine into RNA was maximum after 2 days while for the incorporation of [^{3}H]-thymidine was maximum after 3 days. The optimum time of incubation used for all 5 assays was 3 days for convenience of assaying, if however, 2 days had been used for the uridine assay then there may have been correlation. Also even though uridine uptake is increased in stimulated cells, it is, however not

essential for proliferation and may be blocked with inhibitors without affecting DNA synthesis (Peters and Hausen, 1971). In contrast the inhibition of DNA or protein synthesis, or absence of glucose from the medium all prevent stimulation. Another explanation for the uridine results is that stimulated cells synthesise a high proportion of both ribosomal and non-ribosomal RNA which is metabolically unstable and cannot be recovered (Ling and Kay, 1975).

Glucose is very crucial to activated cells and no stimulation occurs in media devoid of glucose. The lack of correlation between the glucose uptake test and lactate release was most likely due to experimental differences inherent to the assays employed. In the glucose consumption test the amount of glucose removed from the medium by stimulated cells was measured. This will not only include glucose used up in glycolysis (lactate release) but also, glucose stored in the cells as glycogen. In addition, glucose provides many of the intermediates needed for nucleic acids, carbohydrates and protein synthesis (Fodge and Rubin, 1973). In the lactate assay the amount of lactate released into the medium by stimulated cells is measured. This is not necessarily equivilant to the glucose removed from the medium. However, in the lactate release assay, lactate was released into the medium initially, but at later times there is re-uptake of lactate by the cells which was more significant as time increased (Wang et al, 1976). This was the most likely reason for the lack

of correlation. This point was well illustrated by the time-response profiles (Section 3.1.5.). Glucose consumption increases steadily with time till day three then increases slowly. While lactate released into the medium increases sharply reaching a maximum between day 2-3 then declines afterwards. Similar results were found by Wang et al (1976) in their system.

In retrospect it would have been better to measure the ability of stimulated lymphocytes to convert glucose into lactate by using ^{14}C-glucose as a function of time rather than actually measuring the amount of lactate released.

It is difficult to compare the results of this study with reports by other groups of researchers as the details of the assays are often not reported or vary from the ones used here. Hoffman et al (1978) found that, in response to PHA and Con A, 2 out of 11 Guamanian MND patients had decreased responses, as measured by [^{3}H]-thymidine incorporation, and in the same study 3 out of 3 non-Guamanian MND patients showed a normal response. A significantly depressed mean response by lymphocytes of 9 MND patients to PHA was reported by Behan (1979) as measured by [^{3}H]-leucine incorporation but not with [^{3}H]-thymidine incorporation.

More recently, Bartfeld et al (1982) found that the mean responses of PBL from 96 MND patients, tested with PHA or PWM, were comparable to those of age-matched controls; [^{3}H]-thymidine uptake was followed. Antel et al (1982) found similar results with the Con A and PHA reponses of 11 MND patients.

However, the results of this study with non-specific mitogens give evidence for immune cell abnormalities in MND patients.

The investigation into MS was undertaken to study the response of MS PBL to mitogens under the previously stated conditions to observe if a depressed response would be measured as in the case of MND PBLs. MS is a disease with a possible autoimmune cause. The nature of the MS peripheral blood response to various mitogens is still unclear. Many reports found it not to differ from that of normal control populations, or of other patients with neurological diseases. The mitogens investigated were Con A, PHA, PWM, and purified protein derivative (from tuberculin mycobacteria) and the clinical disease activity was also found not to affect the lymphocyte response .

Some studies have, however, shown a decrease in the PBL response to mitogens in MS. A few of these studies were confined to older patients who had had the disease for many years. In other studies the decrease was found when the MS patients were compared to healthy normals but not with neurological diseases (See Introduction Section 2 for more details).

The response observed in this study was compared to normal healthy controls, age and sex matched, as there were no available

samples of the correct age with neurological disease. The stimulation by Con A and PHA of lymphocytes from 10 MS patients was found to be significantly depressed as measured by the [^{3}H]-thymidine, [^{3}H]-leucine, glucose and lactate assays. The decreases probably result from a defect in the T-cell immune functions, possibly the T-cell suppressor subset, which has been reported to be decreased when the disease is active (Huddleston et al, 1979; Huddleston et al, 1982).

4.2. Neuronal Membranes : Stimulation of Lymphocytes

4.2.1. The Morphology and Characteristion of Rat Spinal Cord Cells

The conditions used for the rat spinal cord cell cultures enable the cells to grow and remain viable for 3 weeks. The cultures contained low numbers of contaminating non-neuronal cells as shown in Figure 3.11 (d); a result attributable to the use of serum-free medium from day 3 onwards. The serum-free medium used here was based on that of Bottenstein and Sato (1979) and as modified by Digby et al (1985).

The prior addition of serum-supplemented medium to the tissue plates for 1 h and its use for the first 3 days of culture was essential for cell attachment. Similar requirements for serum have been reported by others (Faivre-Bauman et al, 1961; Bottenstein et al, 1980; Messer et al, 1981) for successful cell culture. This initial period of culture in serum-supplemented

medium was also essential for the long term culture of the cells. The lack of serum in the early days results in early cell death. The growth and migration of cells was rapid and neuronal clusters were seen, rather than individual neurones.

Confirmation of the identity of neurones was achieved by their specific labeling with mouse antineurofilament antibodies and tetanus toxin. Tetanus toxin is a marker for neurones (Mirsky et al, 1978; Vulliamy et al, 1981). Although the neuronal class could not be specified. The presence of cholinergic neurones in such cultures was confirmed by Digby et al (1985) with the measurement of the activity of enzymes choline acetyltransferase and cholinesterase of which the former is believed to be a marker for cholinergic neurones (Gombos et al, 1982) and is present in the motor neurones of the anterior horns (Weil et al, 1977). The levels of both of these enzymes reached maximum values between days 15-20 of culture. Glycine receptors has also been reported (Digby et al, 1984) to increase in parallel with the maturation of these cultures providing further evidence for the presence of neuronal cells.

4.2.2. Stimulation of MND Lymphocytes with membranes from Rat Spinal Cord Cell Cultures

The direct stimulation of lymphocytes with membrane fragments from these cultures resulted in the lymphocytes from 4 out of 14 MND patients showing stimulation indices of greater

than 3, in contrast to 0 out of 9 age-matched controls. These results were obtained with the [^{3}H]-thymidine, [^{3}H]-leucine, [^{3}H]-uridine and lactate assays.

These results indicate that some MND lymphocytes are sensitized to neural membrane components. Bartfeld et al (1982) found that the LMIF responses of MND leucocytes to subcellular brain antigens were significantly inhibited for MND cells exposed to either frontal lobe fraction P1 (nuclei, cell debris and membrane fragments) or fraction P2 (synaptosomes).

These MND-specific responses, together with the reported anti-nuclear antibodies in sera are suggestive of a specific immune response in the disease.

4.2.3. Use of Serum-Free Medium for Peripheral Blood Lymphocytes

The use of serum-free medium for lymphocyte stimulation studies was investigated as a preliminary study to the culture of lymphocytes in Petri dishes already covered with a monolayer of rat spinal cord cells (15 day old). Lymphocyte tissue culture media are commonly supplemented with serum (Forsdyke, 1973; Hirsberg and Thorsby, 1975). However the use of serum introduces many variables such as different serum lots, sources (Hayry and Defendi, 1970) and concentrations (Melchers and Andersson, 1974). Serum can even inhibit growth of lymphocytes (Ivanyi et al,1973) and components of the serum can react with substances whose effects on lymphocytes have been studied (Yachnin et al,1980;

Needleman et al, 1981).

The rat spinal cord cells are only cultured in serum-supplemented medium (SSM) for the initial 3 days. After that period the medium is changed to serum-free medium (SFM). Otherwise the rat spinal cord cells become overgrown with fibroblasts (Digby et al, 1985).

Several serum-free tissue culture media have been reported for culturing lymphocytes from human (Spieker-Polet, 1976; Arai et al, 1977; Hsia et al, 1979; Needleman and Weiler, 1981) mouse (Iscove and Melchers, 1978) and chicken (Kirchner and Oppenheim, 1972) and hybridoma cell lines (Chang et al,1980; Farrant et al, 1981). There have, however, been only two reports of culture of B-cells in serum-free medium; one was in the mouse (Iscove and Melchers, 1978) and the other (Tanno et al, 1982) in human peripheral blood lymphocytes surviving and maturing to IgG and IgM secretion. The latter group replaced fetal calf serum with albumin, transferrin, insulin and fibronectin and this was sufficient for extensive B-cell growth.

The serum-free medium used in the present study was the lymphocyte culture medium without fetal calf serum but supplemented with bovine serum albumin; human transferrin; insulin; biotin; thyroxine; hydrocortisone; sodium selanite; putresceine and progesterone. This medium was very similar to that used by Digby et al (1986).

Whereas neither Con A nor PHA gave reasonable stimulation,

PWM gave a stimulation comparable to that with medium containing fetal calf serum. These results can be explained if serum-free medium supports B-cell growth rather than T-cell growth (Tanno et al,1982).

4.2.4. Coculture of MND Lymphocytes with Rat Spinal Cord Cell Cultures

The sensitisation of PBL from MND lymphocytes to rat spinal cord membrane fragments indicated that there may be some component on the membrane that was responsible for an immune response. The suggestion that the membranes might be contaminated and responsible for this sensitisation prompted the investigation into the co-culture of the rat spinal cord cells and the MND PBL. Serum-free medium was used for the co-culture of MND lymphocytes with rat spinal cord cells. The stimulation was measured by the incorporation of [^{3}H]-thymidine, [^{3}H]-uridine and [^{3}H]-leucine into DNA, RNA and protein respectively. When the lymphocyte number was 1×10^{6} /ml the stimulation was significant in the case of MND patients when compared to age and sex matched healthy controls. This was the case with all the MND patients investigated indicating that only these lymphocytes had become sensitised to a particular component on the rat spinal cord cell surface. The serum-free medium was not favourable for T-cell responses, and this observation indicates that the sensitisation

is as a result of B-cells.

4.3. Monoclonal Antibody Studies

The study of the MND lymphocyte response to rat spinal cord membrane fragments showed that lymphocytes from 4 out of 14 MND patients gave stimulation indices of greater than 3. This was in contrast to 0 out of 9 age-matched controls, as measured by uptake of thymidine, uridine, leucine or lactate release and that 3 out of 3 MND patients lymphocytes showed stimulation indices of greater than 3 when directly cultured with rat spinal cord cells in culture (see Results Section 3.1.12). In view of these findings and those previously reported by Digby et al (1984, 1985) in our department that serum from MND patients was shown to contain antibodies that bind specifically to rat spinal cord cells in culture seems to suggest that there may be an immune response to neural component(s) in MND patients.

The maintenance and development of many autoimmune disorders relies an autoantibodies. There are many examples of this, such as the acetyl choline receptor in myasthenia gravis, IgG in rheumatoid arthritis and nuclear components in systemic lupus erythematosus. The majority of these autoreactive antibodies are polyclonal, which hinders investigation into their structure, specificity, and pathogenic role. In the present investigation

into MND as a possible autoimmune disorder, autoantibodies could provide the key to the precise nature of the antigen or antigens. In myasthenia gravis, the identification of the acetyl choline receptor as the autoantigen was a very important finding, enabling increased understanding of its aetiology and pathogenesis (Harrison and Behan, 1986). If one could characterise the autoantibodies in MND then the auto reactive antigen may be revealed, giving valuable information about the nature of the disease.

This induced the investigation into human monoclonal antibody production from MND patients and the hybridoma technique of Kohler and Milstein (1976) gave the opportunity to study antibody production in MND.

This procedure would provide monoclonal antibodies instead of polyclonal, with no problems of heterogeneity yielding large and inexhaustive supplies. Also when investigating the immune response the availability of a battery of monoclonal antibodies would enable the primary antigen to be distinguished from the secondary in the disease. The detection of the antigen would be made easier hence enabling its possible purification by immunoaffinity. Once the antibody responsible for the disease is detected and purified then there may be a possible diagnostic value in the production and use of anti-idiotypic antibodies.

Human hybridomas are harder to make than rodent ones. Only a few papers have reported the production of human-human hybridomas

and relatively few deal with technical details (see Introduction Section 5). The human cell lines used in human-human fusions are usually Epstein-Barr virus negative. This is because the Epstein-Barr antigen transforms B-cells and it is not desirable to have fused and transformed cells together because one cannot differentiate between the two. There are no commercially available non-secreting human myeloma or lymphoblastoid cell lines.

There are many technical aspects to be taken into consideration, including the myeloma cell line used, the fusion method, selection of hybrids and the identification of antigen-reactive hybrids, subcloning and finally large scale production.

Several different procedures were investigated for the production of human monoclonal antibodies with PBL from MND patients, MS patients and normal healthy controls. The first of which was the direct fusion of human lymphocytes with a non-immunoglobulin secreting mouse myeloma cell line due to the lack of a reliable non-secreting human myeloma cell line. The production of human-human hybridomas was not attempted.

The choice of myeloma cell line was straight forward. The mouse myeloma X63-Ag8.653 was chosen as it does not secrete immunoglobulin and is easily maintained in culture with a doubling time of 18-20 hours (Kearney et al, 1979). The use of a secreting myeloma cell line would result in hybrids producing

mixed immunoglobulins and would only hinder the investigation.

The fusion protocol has been outlined in the Methods Section 2.2.9. However the best results were obtained when the media were warmed to 37°C and all the steps of the fusion were carried out at 37°C. Human peripheral blood lymphocytes are very susceptible to cell death when not kept at this temperature. A ratio of 1:1 human peripheral blood lymphocytes to mouse myeloma was found to give good results (Olsson et al, 1983). Many different PEGs are used in fusions, all with different molecular weights. The toxicity of PEG 4000 was tested and this was subsequently used.

The MND fusions produced 50% of wells with growth and 18% of these were antigen-reactive secreting 80% IgG and 18% IgM. The MS fusions produced 11% of wells with growth of which 4% were antigen-reactive secreting 75% IgG and 15% IgM. The normal fusions produced 50% of wells with growth, secreting 80% IgG and 15% IgM.

The second method was the prior stimulation of the peripheral blood lymphocytes <u>in vitro</u> with PWM before fusion. Astaldi et al, (1981) and Olsson et al, (1983) reported that this gave optimal recovery of antibody producing cells, increasing the numbers of B-cells actively dividing when the cells were stimulated over 5 days. This protocol was undertaken because of the low numbers of B-cells recovered from peripheral blood about 30% of the total number of cells and even lower numbers due to the age of the MND patients and finally to compare with the

direct fusion of the cells. The blood samples used came again from MND, MS patients and normal healthy controls. On the whole the results were comparable, to the fusions performed without prior stimulation with PWM. There were similar numbers of wells producing colonies (50%) and similar numbers secreting IgG (80%) and IgM (15%). The number of antigen-reactive clones seemed greater in the PWM stimulated fusion but not significantly so. The MND produced 18% antigen-reactive clones; MS, 4% antigen-reactive clones and the normal samples gave no antigen-reactive clones with either assay yet the majority secreted immunoglobulin. The stability and immunoglobulin secretion were also very similar, so, on the whole, the stimulation with pokeweed mitogen did little except increase the number of cells.

The third method employed for the production of monoclonal antibodies was using Epstein-Barr virus (EBV). The greatest advantage of using this system are that it is possible to immortalise a high percentage of antibody-producing cells, with no chemical selection. The main disadvantages are the so-called unstability of the transformed cells. However, many such reports concern bulk cultures and even mouse-mouse hybrids lose their immunoglobulin production under such conditions. Many reports suggest preselection of B-cells with the appropriate, antigen-binding specificity as one of the major steps in the transformation. This is to avoid the possibility that non-antibody producing cells would transform and outgrow the required

cells (Steintz et al, 1978; Steintz et al, 1980; Kozbor and Roder, 1981; Steintz, 1981; Campbell, 1985).

MND, MS and normal peripheral blood lymphocytes were cultured with the Epstein-Barr virus after stimulation with PWM for 5 days as suggested by Olsson et al (1983), to increase the number of B-cells. This was advantageous as the number of PBL isolated from MND patients was often very low because of the age of the patient.

The transformed cells grew more quickly than did the hybridisation clones; growth was observed after only a matter of days and after 2 weeks there were over 90% of wells with growth for MND, MS and normal peripheral blood lymphocytes. There was no preselection as the possible auto-antigen in MND is unknown so the screening of the transformed cells was carried out as quickly as possible before overgrowth could take place. The immmunoglobulin secretion was still low (around 250 ng/ml). This has been shown to be increased when preselection was performed (Steintz et al,1981). There were 50% secreting IgG and 50% secreted IgM. This was different from the mouse-human fusions described above where the majority of hybrids secrete IgG. The number of clones secreting the desired antibody detected was about 5% of the colonies formed for MND and MS. The majority were recloned by limiting dilution rather than by cloning in agar (Goding, 1980) because agar gives lower cloning efficiency and the resulting colonies are not always pure clones. The cells were

recloned at cell concentrations of 50, 5, and 0.5 cells/well of which the 5 cells /well gave about 50% of wells with colonies and these were rescreened for positives which were kept in culture in flasks and remained stable for 3 months. The procedure of prior stimulation with PWM increased the number of B-cells but whether the cells should be transformed or fused needs further investigation.

Many of the human secreting cells made immortal by transformation are 'back fused' (Campbell, A.,1985; Thompson et al, 1986). This may only be a safeguard as the reports of loss of secretion are worrying and it is better to be safe than sorry. The back fusion involves the fusion of the desired transformed cell line with either a mouse or human myeloma cell line. Transformed cells fuse exceptionally well as they are growing and replicating fast. These ideas indicate there is still much work to do with the cell lines produced in this research project.

The antibody screening methods employed were mainly enzyme immunoassays. These are safer to use than radioimmunoassays and are more suitable if many screenings are required. The immuno 'dot' assay measured immunoglobulin secretion and gave reasonable results for sensitivity but often it was too sensitive detecting more than one immunoglobulin secreted/well indicating that more than one colony started to grow. This then led to the use of the CELIA which was also sensitve but the results were often

inconsistent from day to day. Finally, the double antibody sandwich ELISA gave the most consistent and sensitive results for measuring antibody class and concentration.

An ELISA was developed for the detection of antigen-reactive colonies in MND and MS. The assay only differed in the coating of the wells. For the MND, membrane fragments prepared from rat spinal cords were used to detect any antibodies directed against spinal cord components. This was chosen because of our finding that MND lymphocytes showed an immune response to rat spinal cord cell cultures and Digby et al (1984, 1985) showed immunoglobulin binding to such cells. The use of actual cultured cells for an ELISA presented too many technical difficulties with the availability of material being limited so the use of adult rat spinal cord membrane fragments was undertaken. This ELISA was rapid and could allow the partial identification of the antigen through absorption assays, i.e., using glycolipids or glycopeptides. In the MS assay the coating was myelin basic protein which is steadily destroyed in MS patients. The results from the clones produced when human peripheral blood lymphocytes were fused directly with the mouse myeloma cell line indicated that the screening methods worked and that there was no cross-reactivity between MS immunoglobulins and MND immunoglobulins. The membrane fragments used in the MND clone screen was purified and probably contained very little myelin basic protein. None of the immunoglobulins produced by the normal clones reacted in

either of the screening methods for colonies reactive with membrane fragments or myelin basic protein. None of these wells were antigen-reactive in each of the screening assays.

The use of florescein-labeled antibodies enabled one to visualise the antibodies under fluorescence optics and the part of the cultured rat spinal cord cells labeled. This method was employed with the antibodies produced by all three previously described methods and from MND, MS and normal healthy controls. There was no clear labeling of the cells by any of the antibodies produced even after the antibodies were concentrated. This may have been due to the antibodies being of low affinity or even after concentrating the amount of relevant antibody was still at too low a concentration.

4.4. Is MND an Autoimmune Disorder?

The etiology in MND is still unknown. There has been intensive research into the disease over the past decade and this has revealed many possibilities as stated in the Introduction. However, because Motor Neurone Disease is a highly specific disorder causing the degeneration predominantly of the motor neurones this makes MND an attractive model an autoimmune disorder. Therefore, the possibility has been investigated for years but many workers report conflicting results when investigating cell-mediated immunity, immune factors, and viral involvement.

The usual indices of abnormality in the immune system, the serum immunoglobulin and complement levels, and peripheral blood cell counts have all been reported normal. The CSF however, is usually normal in these cases but there have been reports of increased protein levels indicative of blood brain barrier damage. Immune involvement in MND is implicated by the detection of immune complexes in sera, spinal tissues and renal tissues. However, the *in vitro* responses to non-specific mitogens are conflicting as various groups of workers find diferent results. The peripheral blood MND B-cell and T-cell counts have been reported to be normal. There have been many reports of potential auto-antibodies but many are controversial. These vary from myelintoxic antibodies, antibodies to neurofilament antigens, and

neurotoxic factors in MND sera, immunoglobulin binding from MND sera to rat spinal cord cells and that these sera recognise a muscle derived protein.

This may take place by attack on the membrane of neurones by antibodies of receptor(s), glycolipids, or glycoproteins. There may even be antibodies to a trophic factor preventing the maintenence and development of the motor neurones.

The results from this study show a depressed response of MND lymphocytes to PHA and Concanavalin A as compared with normal healthy controls. The controls were age and sex matched indicating the response was not related to age. However, the lack of neurological controls led to the study being compared to normality. Other workers have compared there results with MND PBL to other neurological diseases and no depressed response was observed indicating that the response seen in this study may be a general phenomenon of all neurological diseases and not specific for MND.

The responses of the MND lymphocytes to rat spinal cord cell culture membrane fragments, indicates that they were sensitised to the cells. Also, when the cells were co-cultured sensitisation was indicated suggesting an immune response to neural cells. Whether this immune response is the cause of the disease or just secondary, resulting from the breakdown of the motor neurones releasing neural material is questionable. Possible ways of investigating the auto-antigen was through the production of

monoclonal antibodies. This study was however, inconclusive, monoclonals could be produced and detected against spinal cord membrane fragments but when trying to visualise the cells by immunofluorscence to determine which part of the cells these antibodies were directed, nothing could be observed. This study is worthwhile as the autoantigen once detected could allow the use of experimental animal models for future work.

SUGGESTIONS FOR FUTURE WORK

The two main findings of this study are that MND lymphocytes have a depressed blastogenic response to the mitogens Con A and PHA and that there is significant stimulation of such cells with rat spinal cord membranes compared to normals. The stimulation of MND lymphocytes when cultured with rat spinal cord cells indicates that these cells may be sensitised to neural components. Digby et al (1984, 1985) also reported that there is specific binding of immunoglobulin from MND sera. These results suggest that there is a specific immune response in this disease. This may be primary or secondary to immune attack of another target.

The major way forward is to characterise the target antigens(s) responsible for both the antibody binding and the lymphocyte activation. The antigen(s) may be glycoprotein, glycolipid or even receptors on either the membrane surface or intracellular. The neural membranes used for lymphocyte activation studies should be of human origin rather than rat. Further investigation into the antigen responsible could result from the neural membranes being fractionated into their various membrane consituents e.g. glycolipid or glycoprotein. This being subsequently used in lymphocyte activation of MND patients PBL or antibody binding of MND sera yielding a certain component responsible.

The lipids could be extracted from the membrane fragment preparation by chloroform-methanol mixtures or by ethanol, and

then partitioned in a biphasic chloroform-methanol-water system. Most of the lipids are contained in the lower chloroform-methanol layer whereas the gangliosides and larger neutral glycosphingolipids remain in the upper water-methanol layer. Gangliosides can be separated from uncharged glycolipids on columns of DEAE-cellulose, while further purification of all glycolipids is usually performed on column and thin-layer chromatography.

Membrane glycoproteins can be difficult to obtain in a soluble form free from solubilizing agent without denaturation or aggregation. Some however, can be obtained in water soluble forms e.g. glycophorins. When like glycophorins membrane glycoproteins can be extracted into aqueous solution, then further purification can be carried out by standard techniques of protein chemistry. Many glycoproteins can only be extracted in the presence of detergent (triton, sodium dodecyl sulphate), organic solvents, or chaotrophic agents. Therefore procedures have been developed for further fractionation in the presence of the solubilising agents such as affinity chromatography using lectins and ion-exchange.

The production of human monoclonals from MND patients could enable one to identifiy the target antigen(s). The fractions of membrane fragments could be used when screening. The use of Epstein-Barr virus transformation of the cells with the prior

stimulation with PWM would allow for a greater number of colonies being produced and growth would be quicker than fusing with a mouse myeloma where growth can take up to 6 weeks. The increase in the production of colonies by this method compared to mouse-human fusions gives a greater chance of obtaining the required clone. After the desired clone has been detected and recloned by limiting dilution then 'back' fusion with a mouse myeloma non-secreting cell line would safeguard against the loss of secretion.

The production of monoclonal antibodies by in vitro methods could be undertaken. The MND peripheral blood lymphocytes would be incubated with the human neural membranes prior to the fusion with a non-secreting mouse myeloma cell line. This would allows the number of antigen-sensitised cells to proliferate prior to the fusion hence increasing the number of cells secreting the desired antibody being fused.

The use of serum-free media could be investigated for raising monoclonals. Serum-supplemented media are expensive and large quantities of medium are used from the initial fusion to the completion of recloning when raising monoclonals. It would be extremely benefical if serum-free media could be used. Peripheral blood lymphocytes have been shown in this study to proliferate in culture without serum. Therefore in principle, monoclonals (B-cell) could be established in serum-free media if the myeloma cell line could also be maintained in such media.

The antibody produced by the immortalised, sensitised B-cells from MND patients could then be purified by protein A affinity chromatography and then affinity columns could be made with the relevant monoclonal and enable large quantities of the target antigen to be purified. Once the antigen is produced in reasonable amounts then this could be used to induce an experimental model of the disease.

REFERENCES

Aarli, J. A. (1983) J. Neurol., 229, 137

Abdou,N. I., Lisak, R. P., Zweiman, B., Abrahamsohn, I., and Penn, A. S. (1974) New Eng. J. Med., 291, 1271

Ada, G. L. (1970) Transplant Rev., 5, 105

Adkinson, N. F., Rosenberg, S.A., and Terry, W. D. (1974) J. Immunol., 112, 1426

Aiuti, F. (1975) Clin. Immunol. Immunopathol., 3, 584

Alison, A. C. and Mallucci, L. (1964) Lancet ii, 1371

Allison, A. C. (1977) In : N. Talal (Ed.), Autoimmunity, Academic Press, New York, p 92

Allwood, G., Asherson, G. L., Davey, M. J. and Goodford, P. J. (1971) Immunol., 21, 509

Alpers, B. J. and Farmer, R. A. (1949) Arch. Neurol., 62, 178

Andersson, J., Melchers, F., Galanos, C. and Luderitz, O. (1972) J. Exptl. Med., 137, 943.

Andersson, J., Melchers, F., Galanos, C. and Luderitz, O. (1973) J. Expl. Med., 137, 943

Andersson, V., Bird, R.G., Britton, S., and Palatios, R (1981) Immunol. Rev., 57, 5.

Antel, J.P., Arnason, B.G.W., Fuller, P.C.,and Lehrich, J.R. (1976) Arch Neurol., 33, 423.

Antel, J. P. Weinrich, M., and Arnason, B. G. W. (1978) Neurol. Minneap., 28, 999

Antel, J. P., Arnason, B. G. W. and Medof, M. E. (1979) Ann. Neurol., 5, 338

Antel, J. P., Noronha, A. B. C., Oger, J. J. F. and Arnason, B. G. W. (1982) In : P. Rowland (Ed.), Human Motor Neurone Diseases, Raven Press, New York, p. 395

Arai, S., Yamane, I., Tanno, Y., and Takishima, T. (1977) Proc. Soc. Exp. Biol. Med., 154, 444.

Aran, F. A. (1850) Arch. Gen. Med., 24, 5

Ask-Upmark, E. (1961) Acta. Med. Scand., 170, 717

Astaldi, A. (1968) Texas Reports Biol. Med., 26, 461

Astaldi, G. C. B., Janssen, M. C., Lansdorp, P., Willems, C., Zeijlemaker, W. P. and Oosterhof, F. (1980) J. Immunol., 125, 1411

Astaldi, G. C. B., Janssen, M. C., Wright, E. P. and Zeijlemaker, W. P. (1981) Hybridoma Tech., 10,270

Averdunk, R. (1972) Hoppe-Seyler's Z. Physiol. Chem., 353, 79

Bach, M. and Bach, J. (1981) Int. J. Immunopharmacol., 3, 269

Bahaymanyar, S., Moreau-Dubois, M., Brown, P., Cathala, F. and Gajdusek, D. C. (1983) J. Neurimmunol., 5, 91

Bahaymanyar, S., Liem, R. H., Giffin, J. W. and Gajdusek, D. C. (1984) J. Neuropathol. Exp. Neurol., 43, 369

Baran, M. (1985) Immunol. Clin. Prod. Rev., March, 26.

Bartlett, P. F. (1982) Proc. Natl. Acad. Sci. U.S.A., (1982) 79, 2722

Barry, P.S.I. (1975) Br. J. Ind. Med., 32, 119.

Bartfeld, H., Dham, C., Donnenfeld, H., Jashnani, L., Carp, R., Kascsak, R., Vilcek, J., Rapport, M. and Wallenstein, S. (1982)

Clin. Exp. Immunol., 48, 137

Bartfeld, H., Dham, C. and Donnenfeld, H. (1985) J. Neuroimmunol., 9, 131

Bazin, H., Beckers, A., Deckers, C. and Heremans, J.F. (1972) Eur. J. Cancer, 10, 568.

Bazin, H., Beckers, A., Deckers, C. and Moriame, M. (1973) J. Natl. Cancer Inst., 51, 1359.

Behan, P.O., Behan, W.M., Bell, E., Lannigan, C., McQueen, A., and More, J.A. (1977) Lancet ii, 1176.

Behan, P. O., Behan, W. M. H., Zacharias, F. J. and Nicholls, J. T. (1976) Lancet ii, 984

Behan, P. O. (1979) In : F. Clifford-Rose, Clinical Neuroimmunology, Blackwell, Oxford, p 227

Berger, N. A. and Skinner, A. M. (1974) J. Cell. Biol., 61, 45

Berman, M. A. and Weigle, W. O. (1979) J. Exp. Med., 146, 241

Bernard, M. I., Pace, R. C., Unger, S. W. and Wanebo, H. J. (1980) J. Immunol., 124, 964

Berrih, S., Goud, C., Bach, M. A., Le Brigand, H., Binet, J. P., and Bach, J. F. (1981) Clin. Exp. Immunol., 45, 1

Bloom, J. R. Parlette, G. N. and Tropp, N. (1983) Am. J. Dis. Ch., 137, 1175

Bonduelle, M., Bouygues, P. and Lormeau, G. (1970) Presse Med., 78, 827

Booss, J., Esiri, M. M., Tourtellotte, W. W. and Mason, D. Y. (1983) J. Neurol. Sci., 62, 219

Bornstein, M. B. and Appel, S. H. (1965) Ann. New York Acad. Sci., 122, 280

Bottenstein, J. E., Sato, G. H. (1979) Proc. Natl. Acad. Sci. U.S.A., 76, 514

Boyum, A. (1968) Scand. J. Clin. Invest. Suppl., 97, 77.

Bradbury, A. E. (1979) The Concept of the Blood Brain Barrier. John Wiley, Chichester

Bradley, W. G. and Krasin, F. (1982) In : L. P. Rowland (Ed.), Human Motor Neurone Diseases, Raven Press, New York, p 493

Bradley, W. G. and Krasin, F. (1982) Arch. Neurol., 39, 677

Breland, A. E. (1967) Neurol. Minneap., 17, 1011

Brinkman, C. J. J., Nillesen, W. M., Hommes, O. R., Lamers, K. J. B., and DePauw, B. E. (1982) Eur. Neurol., 21, 366

Brodin, T. L., Olsson, L. and Sjogren, H. O. (1983) J. Immunol. Methods, 60, 1

Brody, J. A., Stanhope, J. M. and Kurland, L. T. (1972) In : R. A. Hornbrook, (Ed.), Contempory Trends : Neurology Problems in Oceania, New Guinea, p 346

Brown, M. C., Holland, R. L. and Hopkins, W. G. (1980) Brain Res., 219, 145

Brown, R. H. and Weiner, H. L. (1984) In : F. Clifford-Rose (Ed.), Research Progress in Motor Neurone Disease, Pitman Press, London, p 349

Bron, D., Finberg, M. B., Teng, N. N. H. and Kaplan, H. S. (1984) Proc. Natl. Acad. Sci. U.S.A., 81, 3214

Buckley, P. J., and Wedner, H. J. (1976) J. Immunol., 120, 1930

Bundey, S., Doniach, D. and Soothill, J. F. (1972) Clin. Exp. Immunol., 11, 321

Burnet, F.M. (1959) The Clonal Selection Theory of Acquired Immunity, Cambridge University Press, England, p 1

Campbell, A.M.G., Williams, E.R., and Pearce, J. (1969) Neurology, 19, 1101.

Campbell, A.M.G., Williams, E.R.,and Barltrop, D. (1970) J. Neurol. Neurosurg. Psychiatry, 33, 877.

Campbell, A. (1984) In : R. H. Burdon and P. H. Knippenberg (Eds.), Laboratory Techniques in Biochemistry and Molecular Biology, Elseveir, New York, p 1

Chang, T.H., Steplewski, Z., and Koprowski, H. (1980) J. Immunol. Methods, 39, 369.

Charcot, J. M. and Joffray, A. (1869) Arch. Physiol. Norm. Path., 2, 354

Childs, L. A., Harrison, R. and Lunt, G. G. (1985) J. Neuroimmunol., 9, 69

Chiorazzi (1982) In : A. S. Fauci and R. E. Ballieux (Eds.), Human B-lymphocyte Function, Activation and Immunoregulation, Raven Press, New York, p 47

Circolo, A., Bianchi, R., Nardelli, B., Rivosecchi-Merletti, P. and E. Bonmassar (1982) J. Immunol., 128, 556

Clark, M., Cobbold, S., Hale, G. and Waldman, H. (1983) Immunol. Today, 4, 100.

Clifford-Rose, F. (1984) (Ed.), Research Progress in Motor Neurone Disease, Pitman Press, London p 1

Conradi, S.,Ronnevi, L.O.,and Vesterberg, O. (1978) J. Neurol. Neurosurg. Psychiatry, 41, 389.

Conradi, S., Ronnevi, L.O.,Nise, G., and Vesterberg, O. (1980) J. Neurol. Sci., 48, 413.

Cooper, H. L. (1977) In : D. O. Lucas (Ed.), Regulatory Mechanisms in Lymphocyte Activation, Academic Press, New York, p 94

Cooper, H. L. and Rubin, A. D. (1965) Blood, 25, 1014

Cordiali- Fei, P., Floridi, A., Apollonj Ghetti, N. C. and Natali, P. G. (1980) Immunol. Commun., 9, 389

Cosimi, A. (1981) New Eng. J. Med., 305, 308

Cote, R.J., Morrissey, D.M., Houghton, A.N., Beattie, E.J., Oettgen, H.F., and Old, L.J. (1983) Prot. Natl. Acad. Sci. U.S.A., 80, 2026.

Crawford, D. H., Harrison, J. F., Barlow, M. J., Winger, L. and Huehus, E. R. (1983) Lancet i, 368

Cremer, N.E., Oshiro, L.S, and Norris, S.H. (1973) Arch Neurol., 29, 331.

Croce, C.M., Linnenbach,A., Hall, W., Steplewski, Z. and Koprowski, H. (1980) Nature, 288,488.

Croce, C.M., Shander, M., Martinis, J., Cicurel, L., D'Ancona, G.G. and Kowprowski, H. (1980) Eur. J. Immunol., 10, 486.

Cunningham, D. D. and Pardee, A. B. (1969) Proc. Natl. Acad. Sci.

U.S.A., 64, 1049

Davidson, N. (1976) In : (Ed.), Neurotransmitter Amino Acids, Academic Press, London, p

Davidson, T. J. and Hartman, H. A. (1981) J. Neuropathol. Exp. Neurol., 40, 193

De Belleroche, J., Recordati, R. and Clifford-Rose, F. (1984) In : F. Clifford-Rose (Ed.), Research Progress in Motor Neurone Disease. Pitman Press, England, p 276

De Cock, W., De Cree, J., Van Warwe, J. and Verhagen, H. (1980) J. Immunol. Methods, 33, 127

De Micco, C., Hassoun, J., Meyer, G. and Toga, M. (1986) J. Neuroimmunol., 11, 301

Digby, J. Harrison, R., Jehanli, A., Lunt, G. G. and Behan, P. O. (1982) Abstract Fifth International Congress on Neuromuscular Diseases, Marseilles

Digby, J., Harrison, R., Jehanli, A. and Lunt, G. G. (1983) In : Stella, A. M. G., Gombos, G., Benzi, G. and Bachelard, A. S. (Eds.), Basic amd Clinical Aspects of Molecular Neurobiology, Milan, Menarini, p. 324

Digby, J., Harrison, R., Jehanli, A., Lunt, G.G., Capildeo, R. and Clifford-Rose, F. (1984) In : F. Clifford-Rose (Ed.), Research Progress in Motor Neurone Disease, Pitman Press, London, p. 368

Digby, J., Harrison, R., Jehanli, A., Lunt, G. G. and Clifford-Rose, F. (1985) Muscle and Nerve, 8, 595

Dighiero, G., Lymberi, P., and Holmberg, D. (1985) J. Immunol., 134, 765

Doolittle, W. F. (1984) Nature, 307, 501

Donnenfeld, H., Kascak, R. and Bartfeld, H. (1984) J. Neuroimmunol., 6, 51

Douglas, S. D. (1972) Transplant Rev., 11, 39

Dropcho, E. J., Richman, D. P., Antel, J. P. and Arnason, B. G. W. (1979) Ann. Neurol., 6, 177

Dropcho, E. J., Richman, D. p., Antel, J. P. and Arnason, B. G. W. (1982) Ann. Neurol., 11, 45 177

Dropcho, E. J., Richman, D. p., Antel, J. P. and Arnason, B. G. W. (1982) Ann. Neurol., 11, 456

Dubrow, R., Pardee, A. B. and Pollack, R. J. (1978) J. Cell Physiol., 95, 203

Duchenne, G. B. (1860) Arch. Gen. Med., 2, 283

Eagleson, K. L. and Bennett, M. R. (1983) Neurosci. Letts., 38, 187

Ebaugh, F. G. and McIntyre, O. R. (1962) Blood, 19, 443

Edgington, T. S. and D'Alessio, D. J. (1970) J. Immunol., 105, 248

Edwards, P.A.W. (1981) Biochem. J., 200, 1.

Edwards, P.A.W., and O'Hare, M.J. (1984) In : P. Beverley (Ed.), Methods in Haematology, Churchill-Livingstone, England, p 1

Ehrlich, P. and Morgenroth, J. (1956) In : Himmelweit, F. (Ed.), The Collected Papers of Paul Ehrlich - 1900, Pergamon Press, Oxford, p 1

Ellis, T. (1982) Transplantation, 33, 317

Ellman, G. L.,Courtney, K. D., Andres, V., Featherstone, R. M. (1961) Biochem. Pharmacol., 7, 88

Epstein, L. B. and Stohlman, F. (1964) Blood, 24, 69

Engel, W. K., Kurland, L. T. and Klatzo, I. (1959) Brain, 82, 203

Engel, W. K., Siddeque, T. and Nicoloff, J. T. (1983) Lancet ii, 73

Farrant, J. and Newton, C, (1981) Clin. Exp. Immunol., 45, 504

Fauci, A. S. and Pratt, K. R. (1976) Proc. Natl. Acad. Sci. U.S.A., 73, 3676

Fazekas De St Groth, S.J. and Scheidegger, D. (1980) J. Immunol. Methods., 35, 1.

Feild, E. J. and Hughes, D. (1969) Brit. Med. J., ii, 1399

Felmus, M.T., Patten, B., and Swanke, L. (1976) Neurol., 26, 167.

Festoff, B. (1980) Med. Hypotheses, 6, 121

Fischbach, M., Roubinian, J. R. and Talal, N. (1978) J. Immunol., 120, 1856

Fodge, D. W. and Rubin, H. (1973) Nature New Biol., 246, 181

Forsdyke, D.R. (1973) Immunology, 25, 583.

Forsgren, A., Svedjelund, a. and Wigzell, H. (1976) Eur. J. Immunol., 6, 207

Foung, S. K. H., Perkins, S., Raubitschek, A., Larrick, J., Lisak, G., Fishwild, D., Engleman, E. G. and Grumet, F. C. (1984) J. Immunol. Methods, 70, 83

Fournie, G. J., Lambert, P. H. and Miescher, P. A. (1974) J. Exp.

Med., 140, 1189

Freedman, M. H.,and Khan, N. R. (1979) Can. J. Biochem., 57, 1344

Freidman, A. P. and Freedman, D. (1950) J. Nerv. Ment. Dis., 3, 1

Frey, H. and Molnar, G. (1977) Acta Neurol. Scad., 55, 443

Galfre, G., Milstein, C. and Wright, B. (1979) Nature, 277, 131.

Gardner, J., Chiu, A., Maki, N., and Harris, J.F. (1985) 85, 335.

Garraud, O. and Moreau, T. (1984) J. Immunol. Methods., 75, 95

Garruto, R. M., Gajdusek, D. C. and Chen, K. M. (1981) Ann. Neurol., 10, 341

Garruto, R. M., Yanagihara, R. and Gajdusek, D. C. (1985) Neurol., 35, 193

Gibbs, S. J., Gajdusek, D. C. (1972) J. Clin. Pathol., 25, 132

Giddings, S. J. and Young, D. A. (1974) J. Cell Physiol., 25, 132

Ginsburg, W. W., Frinkleman, F. D. and Lipsky, P. E. (1978) J. Immunol., 120, 33

Goding, J.W. (1980) J. Immunol. Methods, 39, 285.

Goldsby, R. A., Osborne, A., Suri, A., Mandel, J., Williams, E., Gronowicz, E. and Herzenberg, L. A. (1978) Curr. Top. Microbiol. Immunol., 81, 149

Gombos, G., Aunis, D., (1982) Scand. J. Immunol., 15, 317

Gotoh, F., Kitamura, A., Koto, A., Kataoka, K. and Atsuji, H. (1972) J. Neurol. Sci., 16, 201

Goust, J. M., Hoffman, P. M., Pryjma, taoka, K. and Atsuji, H. (1972) J. Neurol. Sci., 16, 201

Goust, J. M., Hoffman, P. M., Pryjma, J., Hogan, E. L. and Fundenberg, H. H. (1980) Ann. Neurol., 8, 526

Greaves, M. and Janossy, G. (1972) Transplant Rev., 11, 87

Green, D. R., Flood, P. M. and Gerson, R. K. (1983) Ann. Rev. Immunol., 1, 439

Gronowitz, E. and Coutinho, A. (1974) Eur. J. Immunol., 4, 776

Gupta, S. (1982) Immunol. Letts., 4, 129

Gupta, S. and Good, R. (1980) Semin. Hematol., 17, 1

Gurney, M. E. and Apatoff, B. (1984) Soc. Neurosci. Abstr., 10, 1051

Gurney, M. E., Belton, A. C., Cashman, C. and Antal, J.P. (1984) N. Engl. J. Med., 311, 933

Gustafson,A. and Stortbecker,P. (1972) Neurol. Minneap., 22, 528

Haahr, S., Moller-Larsen, A. and Pederson, E. (1983) Clin. Exp. Immunol., 51, 197

Hallgren, H. M. and Yumis, E. J. (19) J. Immunol., 118, 2004

Haselbacher, G. K. (1979) Febs. Letts., 100, 185

Hardy, D. A. and Ling, N. R. (1973) In : M. Balls and S. Billet (Eds.), The Cell Cycle in Development and Differentiation, Cambridge University Press, England, p. 397

Harms-Ringdahl, M. and Cooper, H. L. (1978) J. Cell Physiol., 97, 253

Harrison, R., Jehanli, A., Lunt, G. G., and Behan, P. O. (1982) J. Neuroimmunol., 1, 526

Harrison, R. and Behan, P. O. (Eds.) (1986) Clinical

Neurochemistry Vol 1, Academic Press Inc., London, p 59

Hartog, M., Cline, M. J., and Grodsky, G. M. (1967) Clin. Exptl. Immunol., 2, 217

Hausen, P., Stein, H., and Peters, H. (1969) Eur. J. Biochem., 9, 542

Hauser, S. L., Knippers, R. H., Schafer, K. P., Sons, W. and Unsold, H. J. (1976) Exp. Cell Res., 102, 79

Hauser, S. L., Reinhertz, E. L., Hoban, C. J., Schlossman, S. F. and Weiner, C. (1981) Neurol. Minneap., 33, 575

Hauser, S. L., Aubert, C., Burks, J. S. C., Kerr, C., and Lyon-Caen, O. (1986) Nature, 322, 176

Hawkes, C. H., Cavanagh, J. B., Mowbray, S. and Paul, E. A. (1984) In : F. Clifford-Rose (Ed.), Research Progress in Motor Neurone Disease, Pitman Medical Publishing Co. Ltd., England, p

Hayashi, H., Suga, M., Satake, M. and Tsubaki, T. (1981) Ann. Neurol., 9, 292

Haynes, B. (1981) Immunol. Rev., 57, 127

Hayry, P. and Defendi, V. (1970) Clin. Exp. Immunol., 6, 345.

Helderman, J. H., Reynolds, T. C. and Strom, T. B. (1977) Eur. J. Immunol., 8, 589

Heshmati, H. M., Taleb, M. and Turpin, G. (1984) Ann. Endocrinol., 45, 115

Hsia, S., Wilkinson, R.S., and Amos, D.B. (1979) J. Immunol. Methods, 27, 383.

Henle, G. and Henle, W. (1979) In : M. Epstein and B. Achong, (Eds.), Springer-Verlag, Berlin and New York, p 66

Hirano (1976) Neurol. Med., 4,43

Hirano, T., Kuritani, T., Kishimoto, T. and Yamamura, Y. (1977) J. Immunol., 119, 1235

Hirschberg, H. and Thorsby, E. (1975) Cell Immunol., 16, 115.

Hoffman, P. M., Robbins, D. S., Gibbs, C. J. and Gadjusek, D. C. (1977) Lancet ii, 717

Hoffman, P. M., Robbins, D. S., Nolte, M. T. L., Gibbs, C. J. and Gajdusek, D. C. (1978) N. Engl. J. Med., 229, 680

Hoffman, P. M. and Robbins, D. S. (1981) Neurol., 39, 679

Holmberg, D., Forsgren, S., and Ivars, F. (1984) Eur. J. Immunol., 14, 435

Horwitz, M. D., Engel, W. K. and Chaivin, P. B. (1974) Arch. Neurol., 30, 332

Housley, J., and Oppenheim, J. J. (1967) Br. J. Med. ii, 679

Huang, S. W., Rose, J. W., and Mayer, R. F. (1977) J. Neurol. Neurosurg.. Psychiatry, 40, 1053

Huang, S. W., Yang, J., Mayer, R. F., and Moulton, A. (1981) Ann. N.Y. Acad. Sci., 377, 833

Huang, Y. S., Nestruck, A. C., Barbeau, A., Bouchard, J. P. and Davignon, J. (1978) Canad. J. Neurol. Sci., 5, 149

Hughes, R. A. C., Gray, I., Clifford-Jones, R. and Stern, M. (1977) Proc. R. Soc. Med., 70, 874

Ilonen, J., Reunanen, M., Salmi, A. and Tillikainen, A. (1981) J.

Neurol. Sci., 49, 117

Iscove, N. N. and Melchers, F. (1978) J. Exp. Med., 147, 923.

Ivanyi, L., Challacombe, S. J., and Lehner, T. (1973) Clin. Exp. Immunol., 14, 491.

Insel, R. A. and Merler, E. (1977) J. Immunol., 118, 2009

Janossy, G. and Greaves, M. (1975) Transplant Rev., 24, 177

Jokelainen, M., Tiliainen, A., and Lapinleimn, K. (1977) Tissue Antigens , 10, 259.

Jynouchi, H., Kincade, P. W., Good, R. A. and Fernands, G. (1981) J. Immunol., 127, 1232

Kam-Hansen, S., Fryden, A., Link, H. (1978) Acta. Neurol. Scand., 58, 95

Kaplan, J., Mastrangelo, R. and Peterson, W.D. (1974) Cancer Res., 34, 521

Kaplan, J., Nolan, D. and Reed, A. (1982)

Karpas, A., Kampf, U., Siden, A., Koch, M., and Poser, S. (1986) Nature, 322, 177

Kascsak, R.J., Carp, R.I., Vilek, J.T., Donnenfeld, H., and Bartfeld, H. (1982) Muscle and Nerve , 5, 93.

Kay, J. E., Ahern, T. and Atkins, M. (1971) Biochim. Biophys. Acta., 247, 322

Kay, J. E. and Cooper, H. L. (1965) Biochim. Biophys. Acta., 186, 62

Kearney, J. F., Radbruch, A., Leisgand, B. and Rajewsky, K.

(1979) J. Immunol., 123, 1548

Keightly, R. G., Cooper, M. D. and Lawton, A. (1976) J. Immunol., 117, 1538

Kennes, B., Hubert, C., and Neve, P. (1981) Immunol. Lett., 2, 231.

Kennett, R. H., Denis, K. A., Tung, A. S. and Klinman, N. R. (1978) Curr. Top. Microbiol. Immunol., 81, 77

Kirchner, H., and Oppenheim, J.J. (1972) Cell. Immunol., 3, 695.

Kirchner, H., Tosato, G., Blaese, R.M., Broder, S., and Magrath, I.T. (1979) J. Immunol., 122, 1310.

Kletti, L. S., Marton, J. P., Antel, J. P. and Stephansson, K. (1984) Neurol., 34, 238

Knight, S. C., Lance, E. M., Abbosh, J., Munro, A. and O'Brian, J. (1975) Clin. Exp. Immunol., 21, 23

Knott, E., Livini, E., Zamir, R. and Juritzky, A. (1976) Neurol., 26, 376

Koerner, D. R. (1976) Diabetes, 25, 1055

Koffler, D., Biesecker, G. and Katz, S. B. (1982) Arthritis Rheum., 25, 858

Kohler, G. and Milstein, C. (1975) Nature, 256, 495.

Kohler, G. and Milstein, C. (1976) Eur. J. Immunol., 6, 292.

Kondo, K. and Tsubaki, T. (1981) Arch. Neurol., 38, 220

Koprowski, A. (1986), Nature, 322, 176

Kott, E., Livini, E., Zamir, R., and Kuritzky, A. (1976) Neurol., 26, 376.

Kott, E., Livini, E., Zamir, R., and Kuritsky, A. (1979) Neurology, 29,1040.

Kozbor, D., and Roder, J.C. (1981) J. Immunol., 127, 1275.

Koziner, B. and Bloch, K. J. (1976) Ann. New York Acad. Sci., 274, 421

Kurent, J.E., Brookes, B.R., Madden, D.L.,Sover, J.L., and Engel, W.K. (1979) Arch. Neurol., 36, 269.

Kurland, L. T. (1958) J. Chronic Dis., 8, 378

Kurland, L. T., Choi, N.W. and Sayre, G. P. (1969) In : Kurland, L. T. and Norris, F. H. (Eds.), Motor Neurone Diseases, Grune and Stratton, New York, p 1

Kurland, L. T. (1972) Fed. Proc. 31, 1540

Kurlander,H.M.,and Patten, B. (1979) Ann. Neurol., 6, 21.

Kurtze, J.F.,and Beebe, G.W. (1980) Neurol., 30,453.

Lane, D. and Koprowski, H. (1982) Nature, 296, 200

Lane, R.D. (1985) J. Immunol. Methods, 81, 223.

Lagace, J. and Brodeur, B. R. (1985) J. Immunol. Methods, 85, 127

Laurent, P., Beal, B., Gaspar, A., and Guilloux, L. (1985) J. Immunol. Methods, 82, 181.

Lebowitz, S. (1983) In : Batchelor, J. (Ed.), The Immunology of Multiple Sclerosis, Williams and Wilkins, Baltimore, p 379

Lebowitz, S., and Hughes, R. A. C. (1983) (Eds.) In: Immunology of the Nervous System, Academic Press, London, p 10

Lehrich, J.R., Oger, J., and Arnason, B.G.W. (1974) J. Neurol.

Sci., 23, 537.

Leonardi, A., Abbruzzese, G., Arata, L., Cocito, L. and Vishe, M. (1984) J. Neurol., 231, 75

Levy, R., Levy, S., Rosenberg, S. A. and Simpson, R. T. (1973) Biochem., 12, 224

Levy, R. and Kaplan, H. S. (1974) N. Engl. J. Med., 290, 181

Lipsky, P. E., Ginsburg, W. W., Finkelman, F. D. and Ziff, M. (1978) J. Immunol., 120, 902

Lipsky, P. E. and Rosenberg, S. A. (1979) J. Immunol., 122, 926

Limburg, P. C., The, T. H., Hummel-Tappel, E., and Oosterhuis, H. J. G. H. (1983) J. Neurol. Sci., 58, 357

Lindstrom, J. (1979) Adv. Immun., 27, 1

Ling, N. R. and Kay, J. E. (1975) (Eds.), Lymphocyte Stimulation, Elseveir, North Holland, p 1

Lisak, R. P., Awiman, B. and Norman, M. (1975) Arch Neurol., 32, 163

Lisak, R. P. and Zweiman, B. (1977) New Eng. J. Med., 297, 850

Lisak, R. P., Zwiman, B. and Philips, S. M. (1978) Neurol. Minneap., 28, 1298

Lisak, R. P., Smiley, R., Schotland, D. H., Bank, W. J. and Santoli, D. (1979) J. Neurol. Sci., 44, 69

Lisak, R. P., Levinson, R. I., and Zweiman, B. (1982) In : D. L. Schotland (Ed.), Disorders of the Motor Unit, Wiley, New York, p 257

Lisak, R. P., Levinson, R. J. and Zweiman, B. (1985) In : J. M.

Cruse and R. E. Lewis (Eds.), Organ Based Autoimmune Diseases, Karger, Basel, p 65

Littlefield, J.W. (1964) Sci.,145, 709.

Liveson, J., Frey, H. and Bornstein, M. B. (1975) Acta. Neuropathol., 32, 127

Long, W. J., McGuire, W., Palombo, A. and Emini, E. A. (1986) J. Immunol. Methods, 86, 89

Lowrey, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem., 193, 265

Lumsden, C. E. (1972) In : J. Batchelor (Ed.), Multiple Sclerosis a reappraisal, Churchill Livingstone, Edinburgh, p 314

Malave, I., Layrisse, Z. and Layrisse, M. (1975) Cell. Immunol., 15, 231

Mann, D.M.A. and Yates, P. O. (1974) J. Neurosurg. Phsychiatry, 37, 1036

Manaker, S., Shulman, L. H., Winokur, A. and Rainbow, T. C. (1985) Neurol., 35, 1650

Matsumoto, N., Worth, R.M., Kurland, L.T.,and Okazaki, H. (1972) Neurol. Minneap., 22, 934.

Mazzei, D., Novi, C. and Bazzi, C. (1966) Lancet ii, 232

Melamed, M. D., Gordon, J., Ley, S. C., Edgar, D. and Hughes-Jones, N. C. (1985) Eur. J. Immunol., 15, 742

Melchers, F. and Andersson, J.(1974) Eur. J. Immunol., 4, 687.

Mendelsohn, J., Skinner, A. and Kornfeld, S. (1971) J. Clin.

Invest., 50, 818

Miller,J.R., Guntaka, R.V., and Myers, J. (1980) Neurology, 30,884.

Miller, K. B., and Schwartz, R. S. (1982) Adv. Inter. Med., 27,281

Mims, C. (1983) New Sci., 98, 938

Mirsky, R., Wendon, L. M. B., Black, P., Stolkin, C. and Bray, D. (1978) Brain. Res., 148, 251

Mitsuma, T., Nogimori, T., Adachi, K., Mukoyama, M. and Ando, K. (1984) Am. J. Med. Sci., 287, 34

Moller, E., Strom, H. and Al-Balaghi, S. (1981) Scand. J. Immunol., 12, 177

Moretta, L., Webb, S. R., Grossi, C. E. Lydyard, P. M. and Cooper, M. J. (1977) J. Exp. Med., 146, 184

Morimoto, C. (1983) In : B. Haynes and G. Eisenbarth (Eds.), Monoclonal Antibodies, Academic Press, New York p 1

Moser, R. P., Robinson, J. A. and Prostko, E. R. (1976) Neurol., 26, 276

Mshana, R. N., Haregeuo, A. and Belehu, A. (1982) J. Clin. Immunol., 2, 69

Mueller, P. S. and Quick, D. T. (1970) J. Lab. Clin. Med., 76, 190

Mulder, D. W. (1957) Mayo. Clin. Proc. 32, 427

Mulder, D. W. and Howard, F. M. (1976) Mayo. Clin. Proc. 53, 173

Mulder, D.W., Rosenbaum, R.A.,and Layton, D.D. (1972) Mayo. Clin.

Proc., 47, 756.

Murai, A., Miyahara, t., Tanaka, T., Kaneko, T., Sako, Y. and Kameyama, M. (1983) Tohoku J. Exp. Med., 139, 365

Murphy, J. B. and Sturm, E. (1923) J. Exp. Med., 33, 183

Nagano, Y., Tsubaki, T. and Chase, T. N. (1979) Arch. Neurol., 36, 217

Namba, T., Nakata, Y. and Grob, D. (1976) Ann. N.Y. Acad. Sci., 274, 493

Needleman, B.W. and Weiler, J.M. (1981) J. Immunol. Methods, 44, 3.

Needleman, B.W., Weiler, J.M., and Feldbush, T.L. (1981) J. Immunol., 126, 1586.

Nilsson, K. (1971) Int. J. Cancer, 8, 432

Nilsson, K., and Klein, G. (1982) Adv. Cancer Res., 37, 319.

Nobile-Orazio, E., Spagnol, G., and Scarlato, G. (1986) J. Neuroimmunol., 11, 165

Nordal, H. J., and Froland, S. S. (1978) Clin. Immunol. Immunopath., 9, 87

Norris, F. H. and Engel, W. K. (1965) In : W. R. Brain, and F. H. Norris (Eds.), The Remote effects of Cancer on the Nervous System, Grune and Stratton, New York p 24

Norris, F.H. (1975) In : W. G. Bradley and D. Gardner-Medwin (Eds.), Recent Advances in Myology, Excerpta Medica., Amsterdam, p 522.

Norris, F. H. and Engel, W. K. (1979) In : W. R. Brain and F. H. Norris (Eds.), The Remote Effects of Cancer on the Nervous System, Grune and Sratton, New York, p 24

Nowinski, N., Berglund, C., Lane, M., Lostrom, I. (1983) Sci., 210, 537

Novagrodsky, A. and Katchalski, E. (1973) Proc. Natl. Acad. Sci. U.S.A., 70, 1824

Nurcombe, V., Hill, M. A., Eagleson, K. L. and Bennett, M. R. (1984) Brain Res., 219, 19

Obatak, K., Veki, A. and Yoshida, M. (1976) Proc. Japan Acad., 52, 397

Oldstone,M.B.A, Wilson, C.B., Perrin, L.N., and Norris, P.N. (1976) Lancet ii, 169.

Olsson, L., Kronstrom, H., Hosik, C. and Jakobsen, B. (1983) J. Immunol. Methods, 61, 17

Olsson, L. and Kaplan, H. S. (1980) Proc. Natl. Acad. Sci. U.S.A., 77, 5429

Oppenheim, J. J. and Schecter, B. (1976) Lymphocyte Transformation p 81

Palo, J., Rissanen, A., Jokinen, E., Lahdevirta,J., and Salo, O. (1978) Lancet i, 1270.

Parkes, A. B. and Howells, S. (1975) Biochem. Biophys. Res. Commun., 64, 1231

Patten, B. M., Harati, Y., Acosta, L., Jung, S. S. and Felmus, M. T. (1978) Ann. Neurol., 3, 305

Patten, B. M., Kurlander, H. M. and Evans, B. (1982) Acta. Neurol. Scand., 66, 594

Patten, B. (1984) In : F. Clifford-Rose (Ed.), Research Progress In MND, Pitman Medical Publishing Co. Ltd., U.K., p 189

Peavy, D. L., Shands, J. W., Adler, W. H. and Smith, R. T. (1973) J. Immunol., 111, 352

Pederson, L., Platz, L. and Jersild, P. (1976) Inserm., 58, 78

Pertschuk, L.P., Kim, D.S., Prasad, I., Gupta, J.K., Cook, A.W., and Broome, J.D. (1979) In :P. O. Behan and F. C. Rose, (Ed.), Progress in Neurological Research, Pitman Medical Publishing Co Ltd., England, p 44.

Peters, J. H. and Hausen, P. (1971) Eur. J. Biochem., 19, 502

Philips, J. P., Eremin, O. and Anderson, J. R. (1982) Br. J. Cancer, 45, 61

Pierce-Ruhland, R., and Patten, B. (1980) Ann. Neurol., 7,193.

Pietsch, M.C., and Morris, P.J. (1974) Tissue Antigens 4, 50.

Pintus, C., Ransom, J. H. and Evans, C. H. (1983) J. Immunol. Methods, 61, 195

Pogo, B. G. T., Allfrey, V. G. and Mirsky, A. E. (1966) Proc. Natl. Acad. Sci. U.S.A., 55, 805

Polgar, P. R. (1968) Exp. Cell. Res., 49, 231

Pontecorvo, G. (1976) Somat. Cell. Genet., 1,397.

Porstmann, T., Ternynck, T., and Avrameas, S. (1985) J. Immunol. Meth., 82, 169.

Poskanzer, D.C, Cantor, H.M., and Kaplan, G. (1969) In : F. H. Norris, (Ed.), Motor Neurone Disease, Grune and Stratton, New York, p 286.

Prabhaker, B. S., Seagusa, J. and Onodera, T. (1984) J. Immunol., 133, 2815

Prujansky, A., Ravid, A. and Sharon, N. (1978) Biochem. Biophy. Acta., 508, 137

Raine, C. S. (1984) Lab. Invest., 50, 608

Raison, R.L., Walker, K.W., Halnan, C.R.E., Briscoe, D. and Basten, A. (1982) J. Exp. Med., 156, 1380.

Reading, C. (1982) J. Immunol. Methods, 53, 261

Reddy, M. M. and Goh, K. O. (1976) Neurol. Minneap., 26, 997

Reed, D. M., Torres, J. M. and Brody, J. A. (1975) Am. J. Epidemiol., 101, 302

Reinherz, E. L. and Schlossman, S. F. (1980) N. Eng. J. Med., 303, 125

Reinhetz, E. L. and Schlossman, S. F. (1980) Cell, 19, 821

Robbins, J. H. (1964) Sci., 146, 1648

Roberts, N. J. (1979) Sci., 73,160

Roboz, E. E. and Macrae, D. (1969) In : F. H. Norris and L. T. Kurland (Ed), Motor Neurone Diseases, Grune and Stratton, New York, p 175

Roisen, F. J., Bartfeld, H., Donnenfeld, H. and Baxter, J. (1982) Muscle and Nerve, 5, 48

Roitt, I., Brostoff, J. and Male, D. (1985) In : I. Roitt (Ed.),

Immunology, Gower Medical Publishing, London, p 1

Roos, D. and Loos, J. A. (1970) Biochim. Biophys. Acta., 222, 565

Roos, D. and Loos, J. A. (1973) Expl. Cell. Res., 77, 127

Rowland, L. P. (1982) (Ed.), Human Motor Neurone Diseases, Raven Press, New York, p 1

Roos, R.P., Villa, M.C., Wollman, R.,Hatch, M.H., and Antel, J.P. (1980) Arch. Neurol., 37, 312.

Rosenstreich, D. L., Nowotry, A., Chused, T. and Mergenhagen, S. E. (1973) Infection Immunity, 8, 406

Rubin, A. D. and Schultz, E. (1973) In : F. Daguillard (Ed.), Proc. 7th Leuk. Cult. Conf., Academic Press, New York, p 1

Ruhl, H., Kirchner, H. and Rochert, G. (1971) Proc. Soc. Expl. Biol. Med., 137, 1089

Ryan, J. J., Gress, R. E., Hathcock, K. S. and Hodes, R. J. (1984) J. Immunol., 133, 2343

Sagar, H. J., Allonby, I. D. (1979) J. Neurol. Sci., 43, 133

Sagar, H. H., Davies-Jones, G. A. B., and Allonby, I. D. (1980) J. Neurol. Neurosurg. Phsychiatry 43, 967

Sahashi, K., Engel, A. G., Lambert, E. H. and Howard, F. M. (1980) J. Neuropath. Exp. Neurol., 39, 160

Sakane, T. and Green, I. (1978) J. Immunol., 120, 302

Sample, W. F. and Chretein, P. B. (1971) Clin. Exptl. Immunol., 9, 419

Sar, M. and Stumf, W. E. (1977) Sci., 197, 77

Saxon, A., Stevens, R. H. and Ashman, R. F. (1977) J. Immunol., 118, 1872

Schauf, C. L., Schauf, v., Davis, S. A., Strelkaukas, A. J. and Mizen, M. R. (1977) Neurol. Minneap., 27, 822

Schauf, C. L. and Davis, F. A. (1978) Neurol., 28, 34

Schauf, C. L., Antel, J. P., Arnason, B. G. W., Davis, F. A. and Rooney, M. W. (1980) Neurol., 30, 1011

Schauf, V., Schauf, C. L., Mizen, M. and Davis, F. A. (1981) Acta. Neurol. Scand., 64, 383

Schechter, B., Rosenberg, S. A. and Levy, R. (1973) Cell. Immunol., 6, 261

Scheinberg, L. C., Edelman, F. L., and Levy, W. A. (1964) Arch. Neurol., 2, 248

Schlom, J., Wunderlich, D. and Teramoto, Y.A. (1980) Proc. Natl. Acad. Sci. (U.S.A.), 77, 6841

Schwaber, J. and Cohen, E. P. (1973) Nature, 244, 444

Schwaber, J. F. and Rosen, F. C. (1978) J. Exp. Med., 148, 974

Sela, B., Wang, J. L., and Edelman, G. M. (1976) J. Exp. Med. 143, 665

Senitzer, D. and Freimer, E. H. (1984) Rev. Infec. Dis., 6, 832

Sharon, J., Kabat, E.A. and Morrison, S.L. (1982) Mol. Immunol., 19, 389.

Sheremata, W., Moscarello, M. A. and Lopez, D. (1979) Ann. Neurol., 6, 152

Shoenfeld, Y. and Hsu-Lin, S. C. (1982) J. Clin. Invest., 70, 205

Shou, L., Schwartz, S. A. and Good, R. A. (1976) J. Exp. Med., 143, 1100

Sikora, K., Anderson, t., Philips, J. and Watson, J. V. (1982) Lancet i, 11

Simpson, J.A., Seaton, D.A.,and Adams, R.H. (1964) J. Neurol. Neurosurg. Psychiatry, 27, 536.

Simpson, J. A., Behan, P. O., and Dick, H. M. (1976) Ann. N.Y. Acad. Sci., 274, 382

Sincovirs, J.G., Shirato, E., Gyroky, F. Cabiness, J.R. and Howe, C.D. (1970) In: J. G. Sincours (Ed.) Leukemia Lymphoma, Yearbook Med. Publ., Chicargo, p 1

Slack, J. R., Hopkins, W. G. and Pockett, S (1983) Muscle and Nerve 6, 243

Smith, C. I. E., Aarli, J. A., Hammarstrom, L., and Persson, M. A. A. (1984) Neurol., 34, 1094

Spieker-Polet, H. and Polet, H. (1976) J. Biol. Chem., 251, 987.

Stoller, B. D. (1973) In : M. Sela (Ed.), The Antigens, Academic Press, New York, p 1

Strike, L. E., Devens, B. H. and Lundal, R. L. (1984) J. Immunol., 132, 1978

Sugden, B. (1982) Rev Infect. Dis., 4,1048.

Sumner, J. B. (1919) J. Biol. Chem., 37, 137

Steintz, M., Koskimies, S., Klein, G. and Makela, O. (1978) Curr. Top. Microbiol. Immunol., 81, 156

Steintz, M., Izak, G., Cohen, S., Ehrenfeld, M. and Flechner, I. (1980) Nature, 269, 420

Steintz, M. (1981) In : G. J. Hammerling and F. Kearney (Ed.), Monographs in Immunology, Elseveir, North Holland. p 447

Symington, G. R., MacKay, I. R., Whittingham, s., White, J. and Buckley, J. D. (1978) Clin. Exp. Immunol., 31, 141

Tachovsky, T. G. (1976) Lancet ii, 997

Tanaka, K. and Ichihara, A. (1976) Exp. Cell Res., 99, 1

Tanno, Y., Arai, S., and Takishima, T. (1982) J. Immunol. Methods, 52, 255.

Tavolate, B. F., Licandro, A. C. ans Saia, A. (1975) Eur. Neurol., 13,433

Taylor, C. (1977) J. Immunol. Methods, 17, 81

Terasaki, P. I. and Mickey, M. R. (1975) Transplant Rev., 22, 105

Teng, N. N. H., Lam, K. S. Riera, F. C. and Kaplan, H. S. (1983) Proc. Natl. Acad. Sci. U.S.A., 80, 7308

Theiler, M. (1930) Sci., 71, 367

Toh, B. H., Clarence, J. G., Gadjusek, C. D., Goudsit, J. and Dahl, D. (1985) Proc. Natl. Acad. Sci. U.S.A., 82, 3485

Torelli, U., Grossi, G., Artusi, T., Emilia, G., Attija, I. E. and Mauri, C. (1964) Exptl. Cell. Res., 36, 502

Tosato, G., Magrath., I.T., and Blaese, R.M. (1981) In : K. Resh and H. Kirchner (Ed.), Mechanisms Of Lymphocyte Activation, Elsevier, North Holland. p 621

Tosato, G., Magrath, I.T., and Blaese, R.M. (1982) J. Immunol.,

128, 575

Tosato, G., Yarchoan, R., Pike, S.E., De Seau, V., and Blaese, R.M. (1983) Fed. Am. Soc. Exp. Biol., 42, 411.

Tosato, G., Steinberg, A.D., Yarchoan, R., Heilman, C.A., Pike, S.E., De Seau, V., and Blaese, R.M. (1984) J. Clin. Invest., 73, 1789.

Tosato, G.,and Blaese, M.R. (1985) Adv. Immunol., 37, 99.

Traugott, U., Shevach, E., Chiba, J. and Stone, J. H. (1981) Sci., 214, 1251

Trotter,, J. L., Ferguson, T. F., and Garvey, W. F. (1982) J. Neuroimmunol., 3, 99

Turpin, E., Sharif, A., Wantyghem, J., Goussault, Y., and Bourrillon, R. (1980) Biol. Cellulaire, 38, 115.

Unger, M., Wettergr, A., Clausen, J. (1985) Acta Neurosci., 72, 392

Vandenbark, A. A., Saedeleer, J., Heyligen, H., and Dom, R. (1986) J. Neurol., 8, 103

Van Den Berg and Betel (1972) Exp. Cell Res., 66, 257

Vulliamy, T. and Messenger, A. E. (1981) Neurosci. Letts., 22, 87

Walker, J. D., and Cook, J. D. (1979) Neurol. Minneap., 29, 1341

Warburg, O. (1926) Uber den Stoffwechsel des Tumoren, Spinger-Verlag, Berlin

Waxdal, M. J. and Basham, T. Y. (1974) Nature (New Biol.), 251, 163

Wedner, H. T. and Parker, C. W. (1976) Proc. Allergy, 20, 195

Weil, D. E., Busby, W. H. and Mc Ilwain, D. L. (1977) J. Neurochem., 29, 847

Weiner, H. L., Schocket, A. L. (1979) Neurol. Minneap., 29, 1504

Weiner, L. P. (1980) Arch. Neurol., 37, 129

Westall, R. C., Rubin, R., Neider, J. and Jablecki, C. (1983) Immunol. Lett., 7, 139

Westerwould,R. J., Naipal, A. M. and Harrisson, C. M. H. (1983) J. Immunol., 6, 89

Wettenhall, R. E. H., Slobbe, A. and Higgins, T. J. V. (1976) Biochims. Biophys. Acta., 432, 312

Wettenhall, R. E. H., and Slobbe, A. (1979) Biochem. Biophy. Acta., 56, 400

Whitaker, J. N., Sciabbarrasi, B. S., Engel, W. K., Warmott, J. R. and Strober, W. (1973) Neurol., 23, 1164

Whitehouse, P. J., Wamsley, J. K., Zarbin, M. A. (1983) Ann. Neurol., 14, 8

Whiting, M. G. (1963) Econ. Bot., 17, 270

Whiting, M. G. (1964) Fed. Proc., 23, 1343

Whitney, R. B. and Sutherland, R. M. (1972) Cell. Immunol., 5, 137

Wijermans, P., Oosterhuis, H. J. G. H., Astaldi, G. C. B., Schellekens, P. T. A., and Astaldi, A. (1980) J. Immunol., 124, 1977

Wikstrand, C. J. and Bigner, D. D. (1980) Am. J. Pathol. 98, 515

Williams, C.A. and Chase, M.W. (1967) Methods in Immunology and Immunochemistry, Vol. 1, Academic Press, New York, p 1

Wilson, S.A.K. (1907) Rev Neurol. Psychiat., 5,441.

Wolfgram, F. and L. Myers (1973) Science, 179, 579

Wolfgram, F. (1976) In : Andrews, Johnson, and Bracier (Ed.), ALS Recent Research Trends, Academic Press, New York, p 145

Wool, I. G. and Cavicchi, P. (1966) P. Nas. U.S.A., 56,991

Yachnin, S., Saltani, K., and Lester, E.P. (1980) J. Allergy Clin. Immunol., 65, 127

Yamana, S., Matsueda, H., Ezawa, H., Miyake, S. and Katsuyam, S. (1978) Acta. Med. Oka., 31, 441

Yase, Y., Matsumoto, N. and Yoshimasu, F. (1968) Proc., Aust. Assoc. Neurol., 5, 335

Yase, Y., Matsumoto, N., Yoshimasu, F. and Handa, Y. (1973) Neurol., 20, 531

Yase, Y. (1979) ALS in the Kii Peninsula, One Possible Ethological Hypothesis. In : Tsubaki, T.,and Toyokura, Y. (Ed.), ALS, Baltimore University Press, p 307

Yase, Y. (1980) Neurotoxicology, 1,101

Yasmeen, D., Laird, A. J., Hume, D. A. and Weideman, M. J. (1977) Biochim. Biophys. Acta. 500, 89

Yarchoan, R., Tosato, G., Blaese, R.M., Simon, R.M., and Nelson, D. (1983) J. Exp. Med., 127, 1.

Yen, A. and Stein, L. S. (1981) Cell. Immunol., 57, 440

Yen, A., and Lewin, D. (1981) Cell. Immunol., 61, 332

Yen, A. and Dulgou, R. (1983) Immunol. Letts., 6, 169

Yoshino, Y., Koike, H. and Akaik, K. (1976) Experimentia 35, 219

Yoshino, Y., Koike, H. and Akaik, K. (1979) Experimentia 35, 219

Zaiwalla, Z., Gawel, M. J., Markwick, J. and Clifford-Rose, F. (1984) In : F. Clifford-Rose (Ed.), Research Progress in Motor Neurone Disease, Pitman Medical Publishing Co. Ltd., London, England p 384

Zilkha, K.H. (1962) Proc. Roy. Soc. Med., 55,1028.

Zilko,P. J., Dawkins, R. L., Holmes, K., and Witt,, C. (1979) Clin. Immunol. Immunopathol., 14, 222

Zvaifler, N. J. (1977) In : N. Talal (Ed.), Autoimmunity, Academic Press, New York, p 369

Zvaifler, N. and Bluestein, H. (1982) Arthritis Rheum., 25, 862

PUBLICATIONS

Journal of Neuroimmunology, 11 (1986) 31–40
Elsevier

JNI 00335

Stimulation by Mitogens and Neuronal Membranes of Lymphocytes from Patients with Motor Neurone Disease

Jaqueline Aspin [1], Roger Harrison [1], Ahmed Jehanli [1], George Lunt [1] and Malcolm Campbell [2]

[1] *Department of Biochemistry, University of Bath, Claverton Down, Bath, and* [2] *Neurology Department, Bristol Royal Infirmary, Bristol (U.K.)*

(Received 15 February, 1985)
(Revised, received 10 June, 1985)
(Accepted 17 July, 1985)

Summary

Stimulation of lymphocytes from motor neurone disease patients by either concanavalin A or PHA was shown to be significantly depressed relative to that from normal controls, as assayed by incorporation of [^{3}H]thymidine or [^{3}H]leucine or by glucose uptake. Corresponding significant differences were not shown by assays based upon incorporation of [^{3}H]uridine or of lactate release. Lymphocytes from 4 out of 14 motor neurone disease patients showed a blastogenic reponse to membranes from rat spinal cord cells, compared with those from 0 out of 9 normal controls. These results not only suggest the possibility of an impaired cellular immune control in MND patients but also indicate the presence of lymphocytes sensitised specifically to neuronal membrane components.

Key words: *Concanavalin A – Lymphocyte stimulation – Motor neurone disease – PHA*

This work was supported by the Muscular Dystrophy Group of Great Britain and Northern Ireland. J.A. thanks the S.E.R.C. for a research studentship.

0165-5728/86/$03.50 © 1986 Elsevier Science Publishers B.V. (Biomedical Division)

Introduction

Motor neurone disease (MND) is a chronic progressive disorder characterised by degeneration of the anterior horn cells in the spinal cord and, in some cases, of the pyramidal cells of the cerebral cortex. The pathogenesis and aetiology of the disease are unknown. One aspect that is currently receiving renewed attention is a possible involvement of the immune system; an area in which both clinical and laboratory studies have produced many equivocal findings (for reviews see Rowland 1982; Clifford Rose 1984). However, early reports of a serum-borne neurotoxic factor in MND patients (Wolfgram and Myers 1973) have recently been confirmed by Roisen et al. (1982) and we have shown that MND sera contain immunoglobulins that bind to spinal cord cells in culture (Digby et al. 1984, 1985). Very recently Gurney and his colleagues (Gurney and Apatoff 1984; Gurney et al. 1984) have demonstrated the presence in MND sera of autoantibodies directed against a muscle-derived growth factor for spinal neurones.

Investigations of immune cells in motor neurone disease have produced conflicting data. Thus total T cell count and T cell subsets (suppressor/helper ratios) have been reported unchanged in MND patients (Antel et al. 1982; Bartfeld et al. 1982) compared to normal controls whereas T cell subsets as determined by the expression of receptors for Fcγ and Fcμ were found by Westall et al. (1983) to be significantly different in such patients. Depressed responses to non-specific mitogens have been reported by some workers (Hoffman et al. 1978; Behan 1979) but other groups found no such effects (Antel et al. 1982; Bartfeld et al. 1982). In initial studies, we observed a decreased response of total peripheral blood lymphocytes from MND patients compared with matched controls in assays using non-specific mitogens (Digby et al. 1984). The present study confirms these findings and extends our investigation to an examination of lymphocyte responses to membrane preparations from cultured spinal cord cells.

Materials and Methods

Patients

Eighteen patients (14 males, 4 females) were investigated in this study. The age range was from 40 to 80 years with a mean $\pm$ SD of 61.1 $\pm$ 9.8 years. Diagnosis of MND was based on a combination of both upper and lower motor neurone findings coupled with EMG changes characteristic of denervation. Normal controls (20) were age- and sex-matched as far as possible; the age range was 41–77 years with a mean $\pm$ SD of 55.6 $\pm$ 9 years.

Lymphocyte preparation

Fresh heparinized venous blood was mixed with an equal volume of Dulbecco's Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) (Flow Labs., Irvine, U.K.) and carefully layered onto a Ficoll-Hypaque gradient (Flow Labs.). After centrifugation at 400 $\times$ g for 40 min, the mononuclear cell layer at the interface was carefully

removed and washed 3 times with PBS (20 ml). Cells were resuspended at 1×10^6 cells/ml in RPMI 1640 medium (Flow Labs.), supplemented with 100 units/ml penicillin; 100 μg/ml streptomycin; 2 mM L-glutamine and 10% heat-inactivated foetal calf serum (Gibco, Uxbridge, U.K.). Cells used for [^{3}H]leucine incorporation studies were resuspended in leucine-free Eagle's minimum essential medium (Gibco) supplemented as above.

Mitogen stimulation

Samples (100 μl; 1×10^5 cells) of the lymphocyte cell suspension were placed in the wells of U-bottomed microtitre tissue culture plates (Nunc, Gibco, Uxbridge, U.K.). An equal volume of the complete medium containing the appropriate concentration of concanavalin A, phytohaemagglutinin (PHA) (both from Pharmacia, Hounslow, U.K.) or membrane fragments (see below) was added. Control cultures received complete medium only. Cultures were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C for 72 h. Experiments were performed in triplicate for each preparation.

Glucose consumption assay

This was carried out according to De Cock et al. (1980). At the termination of the culture period, samples (100 μl) of the supernatant were removed from both stimulated and control cultures. The amount of glucose present was measured by using a glucose oxidase assay kit (Boehringer, Mannheim, F.R.G.). Lymphocyte stimulation was expressed as the difference between glucose used by control and stimulated cells.

Lactate assay

This procedure is similar to that described for the glucose assay. The lactate content of samples of supernatant from both control and stimulated cultures was assayed by using a lactate test combination kit (Boehringer, Mannheim). Stimulation was expressed as the difference in the lactate content of the supernatant from the stimulated and control cells.

Incorporation of [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]leucine

At the appropriate time, cultures were exposed to radiolabelled precursor for 18 h. The precursors (0.2 μCi) were added in RPMI 1640 medium (10 μl). Cells were harvested and the acid-precipitable radioactivity was measured in a liquid scintillation spectrometer (Packard Tri-Carb, Packard Instruments, Reading, U.K.).

Lymphocyte stimulation was expressed as log ($cpm_{stimulated} - cpm_{control}$) or as the stimulation index ($cpm_{stimulated}/cpm_{control}$), where control refers to the test sample incubated in the absence of mitogen.

Rat spinal cord cell culture

14- to 16-day-old rat embryo spinal cord cells were cultured as described earlier (Digby et al. 1985). Briefly, a single cell suspension of spinal cords was prepared by mincing and trypsinization. 1.5×10^6 cells were seeded on collagen-coated 3.5-cm

Petri dishes (Nunc, Gibco) in Dulbecco's modified minimum essential medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum (Gibco), 10% heat-inactivated donor horse serum (Flow Labs.), penicillin (100 units/ml), streptomycin (100 μg/ml), 2 mM L-glutamine and glucose (1.5 mg/ml). The cultures were incubated in 10% CO_2/air at 37°C. After 3 days the medium was replaced with a serum-free medium which lacked the serum supplement but contained the following reagents: insulin (0.2 units/ml), transferrin (5 μg/ml), 20 nM progesterone, 100 μM putrescein, 30 nM sodium selenite, 0.5 μM hydrocortisone, biotin (1 μg/ml) and thyroxine (3 μg/ml). All reagents were from Sigma Co., Poole, U.K. This medium (SFM) was subsequently changed every 3 days.

The preparation of crude membrane fractions from spinal cord cultures

Cells from 15- to 17-day-old cultures were scraped from the surface of the Petri dishes using a Teflon-coated spatula and 2 aliquots (50 μl) per dish of 0.3 M sucrose in 5 mM Tris/HCl buffer, pH 8.0. The mixture was homogenised by using a glass homogeniser and centrifuged at $1000 \times g$ for 10 min. The nuclear pellet was discarded and the supernatant was centrifuged at $15\,000 \times g$ for 30 min at 4°C. The supernatant was discarded and the pellet homogenised in 5 mM Tris/HCl buffer, pH 7.4 (20 vols.) by using a glass homogeniser. The mixture was centrifuged at $9000 \times g$ for 20 min at 4°C. The pellet was discarded and the supernatant centrifuged at $48\,000 \times g$ for 20 min. The pellet was resuspended in RPMI 1640 medium at a final concentration of 2 mg/ml and aliquots were frozen at −70°C.

Results

Effects of mitogen concentration and culture time on lymphocyte stimulation

Dose- and time-response curves were measured in order to establish optimum mitogen concentrations and culture times. Stimulation of lymphocytes was assayed in terms of DNA, RNA and protein synthesis by incorporation of radiolabelled thymidine, uridine and leucine, respectively. Lactate production and glucose uptake were also measured as indices of metabolic activity. Lymphocytes from 5 normal controls (age range 30–65 years, mean 52 years) were stimulated with increasing concentrations of concanavalin A and PHA and the stimulation was measured by each of the 5 methods after 3 days in culture. The results showed that the maximum response was observed in all cases with concentrations of mitogen of 10–15 μg/ml. In all subsequent studies 7.5 μg/ml mitogen was used.

In time-response experiments, DNA, RNA and protein synthesis, as measured by the incorporation of the relevant precursor, increased rapidly, reaching maximum values after 2, 3 and 4 days, respectively. The rate of glycolysis, measured by lactate release, was maximal after 3 days in culture. Glucose uptake increased rapidly up to 3 days of culture and thereafter increased slowly. From these experiments, an incubation period of 3 days was chosen for subsequent studies.

MND lymphocytes: effects of concanavalin A and PHA

Peripheral blood lymphocytes from MND patients and from matched controls were stimulated with concanavalin A and with PHA, and the results are shown in

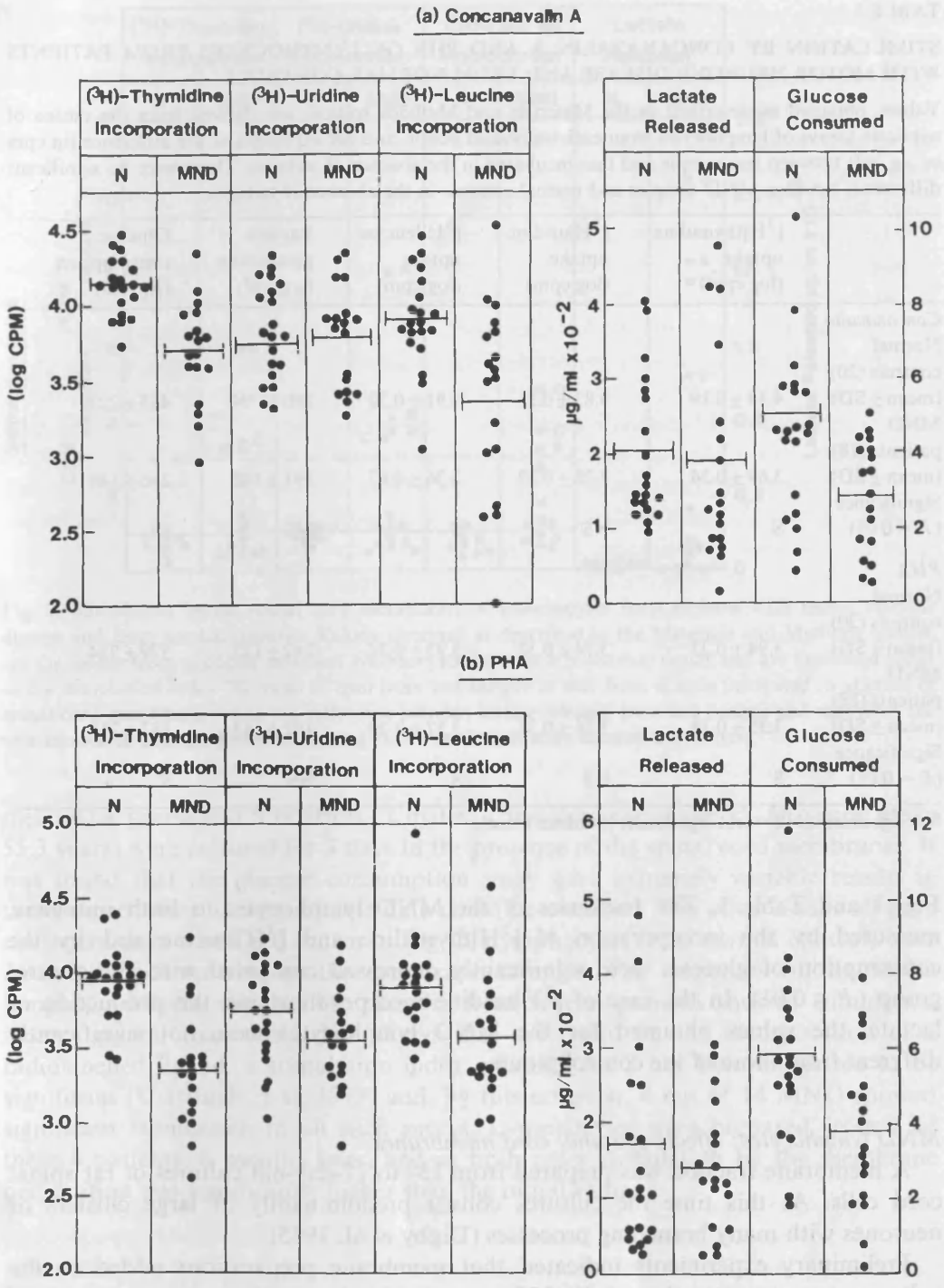


Fig. 1. Stimulation, by concanavalin A (*a*) and PHA (*b*), of lymphocytes from patients with motor neurone disease and from normal controls. Values, obtained as described in the Materials and Methods section, are the means from triplicate assays of lymphocytes from each individual donor and are expressed as the difference (in cpm or μg/ml) between test sample and that incubated in the absence of mitogen.

TABLE 1

STIMULATION BY CONCANAVALIN A AND PHA OF LYMPHOCYTES FROM PATIENTS WITH MOTOR NEURONE DISEASE AND FROM NORMAL CONTROLS

Values, obtained as described in the Materials and Methods section, are derived from the means of triplicate assays of lymphocytes from each individual donor, and are expressed as the difference (in cpm or μg/ml) between test sample and that incubated in the absence of mitogen. There were no significant differences between MND samples and normal controls in the absence of mitogen.

	[^{3}H]thymidine uptake (log cpm)	[^{3}H]uridine uptake (log cpm)	[^{3}H]leucine uptake (log cpm)	Lactate production (μg/ml)	Glucose consumption (μg/ml)
Concanavalin A					
Normal controls (20) (mean ± SD)	4.13 ± 0.19	3.83 ± 0.31	3.91 ± 0.30	200 ± 94	453 ± 221
MND patients (18) (mean ± SD)	3.69 ± 0.34	3.78 ± 0.33	3.36 ± 0.67	191 ± 142	286 ± 148
Significance ($P = 0.01$)	S	NS	S	NS	S
PHA					
Normal controls (20) (mean ± SD)	3.94 ± 0.23	3.74 ± 0.32	3.93 ± 0.32	142 ± 125	579 ± 224
MND patients (18) (mean ± SD)	3.35 ± 0.38	3.56 ± 0.32	3.57 ± 0.39	141 ± 83	382 ± 212
Significance ($P = 0.01$)	S	NS	S	NS	S

S = significant, NS = not significant (Student *t*-test).

Fig. 1 and Table 1. The responses of the MND lymphocytes to both mitogens, measured by the incorporation of [^{3}H]thymidine and [^{3}H]leucine and by the consumption of glucose, were significantly depressed compared with the control group ($P < 0.01$). In the case of [^{3}H]uridine incorporation and the production of lactate, the values obtained for the MND lymphocytes were not significantly different from those of the control group.

MND lymphocytes: effects of spinal cord membranes

A membrane fraction was prepared from 15- to 17-day-old cultures of rat spinal cord cells. At this time the cultures consist predominantly of large clusters of neurones with many branching processes (Digby et al. 1985).

Preliminary experiments indicated that membrane preparations added to the cultures at a concentration of 50–100 μg protein/ml gave the maximum response and a concentration of 50 μg protein/ml was accordingly used for all subsequent experiments.

Lymphocytes from 14 MND patients (11 males, 3 females; age range 44–80 years,

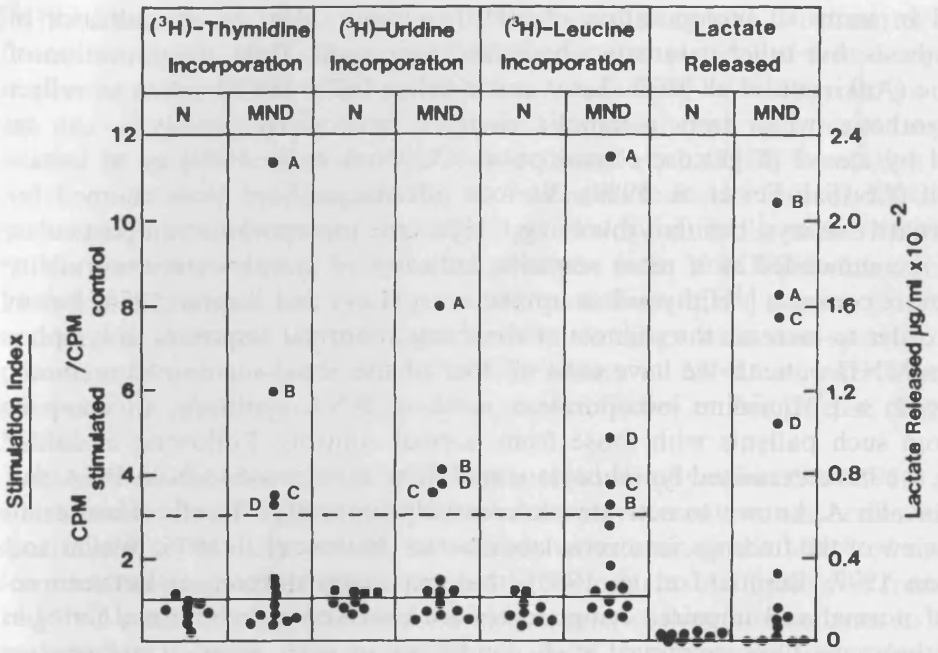


Fig. 2. Stimulation by rat spinal cord membranes of lymphocytes from patients with motor neurone disease and from normal controls. Values, obtained as described in the Materials and Methods section, are the means from triplicate assays of lymphocytes from each individual donor and are expressed either as the stimulation index (SI, ratio of cpm from test sample to that from sample incubated in absence of spinal cord membranes) or as the difference between lactate released from test sample and that from the non-stimulated control. Patients showing SI > 3 are individually identified by letters.

mean 62.4 years) and 9 controls (7 males, 2 females; age range 35–65 years, mean 55.3 years) were cultured for 3 days in the presence of the spinal cord membranes. It was found that the glucose consumption assay gave extremely variable results in these experiments and no reliable data could be obtained. The other assays all gave consistent data which are shown in Fig. 2. Lymphocytes from normal controls were essentially non-reactive to the membrane samples. The responses of the MND lymphocytes were variable but samples from several patients showed stimulation indices greater than unity. For assays that are based on the incorporation of a radiolabelled ligand, a stimulation index greater than 3 has been suggested to be significant (Urbaniak et al. 1978) and, by this criterion, 4 out of 14 MND showed significant stimulation in all such assays. Lymphocytes were prepared from 2 of these 4 patients, 6 months later, and in both cases stimulation by the membrane preparation was significantly higher than the initial value.

Discussion

The response of peripheral blood lymphocytes to non-specific mitogens is commonly used as a measure of cellular immune capability. Blastogenesis is usually

quantified in terms of incorporation of [^{3}H]thymidine, taken as an indicator of DNA synthesis, but other parameters have also been used. Thus, incorporation of [^{3}H]leucine (Adkinson et al. 1974; Levy and Kaplan 1974) can be taken to reflect protein synthesis, while early metabolic changes, particularly glycolysis, can be monitored by assays of glucose consumption (De Cock et al. 1980) or of lactate production (Cordiali-Fei et al. 1980). Various advantages have been claimed for these alternative assays, but that involving [^{3}H]leucine incorporation, in particular, has been recommended as a more sensitive indicator of lymphocyte abnormality than the more common [^{3}H]thymidine uptake assay (Levy and Kaplan 1974; Behan 1979). In order to increase the chances of detecting abnormal responses in lymphocytes from MND patients we have used all four of the above-mentioned methods, together with a [^{3}H]uridine incorporation assay of RNA synthesis, to compare results from such patients with those from normal controls. Following a similar reasoning, we have examined lymphocyte stimulation in response to both PHA and to concanavalin A, known to activate preferentially suppressor T cells (Shou et al. 1976). In view of the findings, in several laboratories (Malave et al. 1975; Waller and MacLennan 1977; Bernhard et al. 1980), that maximum differences between responses of normal and impaired lymphocytes are observed at suboptimal mitogen concentrations, we have employed such conditions in each assay. Time courses indicated that, for all assays, response to mitogen had achieved near maximum values at 3 days, a period which was shown to lead to greatest discrimination between normal and impaired lymphocytes in the studies of Bernhard et al. (1980). This incubation period was consequently chosen for all subsequent comparisons.

The response, to both PHA and concanavalin A, of lymphocytes from 18 MND patients was significantly depressed ($P < 0.01$), relative to normal controls, as measured by incorporation of [^{3}H]thymidine or of [^{3}H]leucine and by glucose consumption.

No significant differences between MND lymphocytes and controls were observed in the cases of [^{3}H]uridine incorporation or lactate production. While the reasons for these latter results are not clear, they are not necessarily inconsistent with our other findings. RNA and DNA synthesis are shown by different proportions of a given population of lymphocytes stimulated by PHA (Ling and Kay 1975) and inhibition of uridine uptake need not affect DNA synthesis (Peters and Hausen 1971), nor is there any reason to expect that uridine incorporation should parallel protein synthesis. A further complicating factor is that a high proportion of RNA synthesised in stimulated cells is metabolically unstable and cannot be recovered (Ling and Kay 1975). Similar arguments apply to the assays based on glucose uptake and lactate production, which need not be closely linked. Whereas uptake of glucose reflects its utilisation in a range of both anabolic and catabolic pathways, lactate production might be expected to indicate specifically glycolytic activity. Moreover, conclusions based on lactate production are complicated by a time-dependent increase in lactate utilisation in stimulated cells.

Comparison with previously reported findings is difficult in that details of the assays vary, or are not reported. Behan (1979) reported a significantly depressed mean response to PHA by lymphocytes from 9 MND patients, as assayed by

[^{3}H]leucine incorporation, but not by [^{3}H]thymidine uptake. Hoffman et al. (1978) found that 2 out of 11 Guamanian MND patients tested had lymphocytes hyporesponsive to both PHA and concanavalin A as judged by [^{3}H]thymidine uptake; 3 out of 3 non-Guamanian patients showed normal lymphocyte responses in these studies. In more recently reported findings, Antel et al. (1982) found that the mean responses to PHA and concanavalin A of mononuclear cells from 11 MND patients were comparable to those of age-matched healthy controls, while Bartfeld et al. (1982) drew similar conclusions for lymphocytes from 96 MND patients tested with PHA or with pokeweed mitogen; in both of these latter studies [^{3}H]thymidine uptake was followed. Overall, our present findings with non-specific mitogens provide evidence for immune cell abnormalities in MND patients that may be relevant to the presence of circulating autoantibodies in this disease (Digby et al. 1985).

In view of the above and previously reported (Digby et al. 1984, 1985) results in which serum from MND patients was shown to contain antibodies that bind specifically to spinal cord cells in culture, we examined the effect of membranes from such cultures on lymphocytes from MND patients. Lymphocytes from 4 out of 14 MND patients showed stimulation indices greater than 3, in contrast to 0 out of 9 age-matched controls, as measured by uptake of thymidine, uridine or leucine or by lactate release. These results are consistent with the sensitisation of MND lymphocytes to neuronal membrane components. Bartfeld et al. (1982) compared the responses of MND and control leycocytes to subcellular brain antigens by means of the leucocyte migration inhibition factor (LMIF) and found significantly greater inhibition for MND cells exposed to either frontal lobe fraction P1 (nuclei, cell debris and membrane fragments) or fraction P2B (synaptosomes). These MND-specific responses to neuronal membranes are supported by our present lymphocyte stimulation data which, together with the demonstration of anti-neuronal membrane antibodies in MND sera, combine to indicate a specific immune response in the disease. Whether an immune response to neuronal membranes is secondary to immune attack at another target, e.g. nerve growth factor (Gurney and Apatoff 1984; Gurney et al. 1984) or incidental to neuronal degenerations caused by other means remains unclear.

References

Adkinson, N.F., S.A. Rosenburg and W.D. Terry, Early detection of lymphocyte stimulation and mixed lymphocyte interaction in man with a semimicro protein synthesis assay, J. Immunol., 112 (1974) 1426–1434.

Antel, J.P., A.B.C. Noronha, J.J.F. Oger and B.G.W. Arnason, Immunology of amyotrophic lateral sclerosis. In: P. Rowland (Ed.), Human Motor Neuron Diseases, Raven Press, New York, 1982, pp. 395–401.

Bartfeld, H., C. Dham, H. Donnenfeld, L. Jashnani, R. Carp, R. Kascsak, J. Vilcek, M. Rapport and S. Wallenstein, Immunological profile of amyotrophic lateral sclerosis patients and their cell mediated immune responses to viral and CNS antigens, Clin. Exp. Immunol., 48 (1982) 137–147.

Behan, P.O., Cell mediated immunity in motor neurone disease and poliomyelitis. In: F. Clifford Rose (Ed.), Clinical Neuroimmunology, Blackwell, London, 1979, pp. 259–272.

Bernhard, M.I., R.C. Pace, S.W. Unger and H.J. Wanebo, The influence of incubation time and mitogen concentration on lymphocyte blastogenic response: determination of conditions that maximise population differences, J. Immunol., 124 (1980) 964–968.

Clifford Rose, F. (Ed.), Research Progress in Motor Neurone Disease, Pitman Press, London, 1984.

Cordiali-Fei, P., A. Floridi, N.C. Apollonj Ghetti and P.G. Natali, Estimation of PHA induced transformation in peripheral blood lymphocytes through measurement of their increased glycolysis, Immunol. Commun., 9 (1980) 389–401.

De Cock, W., J. De Cree, J. Van Warwe and H. Verhagen, Measurement of mitogen stimulation of lymphocytes with a glucose consumption test, J. Immunol. Methods, 33 (1980) 127–131.

Digby, J., R. Harrison, A. Jehanli, G.G. Lunt, R. Capildeo and F. Clifford Rose, Immunological changes in motor neurone disease. In: F. Clifford Rose (Ed.), Research Progress in Motor Neurone Disease, Pitman Press, London, 1984, 368–378.

Digby, J., R. Harrison, A. Jehanli, G.G. Lunt and F. Clifford Rose, Cultured rat spinal cord neurones: interaction with motor neurone disease immunoglobulins, Muscle Nerv., 8 (1985) 595–605.

Gurney, M.E. and B. Apatoff, Activity of a muscle derived growth factor for spinal neurones in vivo and in vitro, Soc. Neurosci. Abstr., 10 (1984) 1051.

Gurney, M.E., A.C. Belton, N. Cashman and J.P. Antel, Inhibition of terminal axon sprouting by serum from patients with amyotrophic lateral sclerosis, N. Engl. J. Med., 311 (1984) 933–939.

Hoffman, P.M., D.S. Robbins, M.T.L. Nolte, C.J. Gibbs and D.C. Gajdusek, Cellular immunity in Guamanians with amyotrophic lateral sclerosis and Parkinsonism-dementia, N. Engl. J. Med., 229 (1978) 680–685.

Levy, R. and H.S. Kaplan, Impaired lymphocyte function in untreated Hodgkin's disease, N. Engl. J. Med., 290 (1974) 181–186.

Ling, N.R. and J.E. Kay, Lymphocyte Stimulation, North-Holland, Amsterdam, 1975.

Malave, I., Z. Layrisse and M. Layrisse, Dose-dependent hyporeactivity to phytohaemagglutinin in systemic lupus erythematosus, Cell. Immunol., 15 (1975) 231–236.

Peters, J.H. and P. Hausen, Effect of PHA on lymphocyte membrane transport. I. Stimulation of uridine uptake, Eur. J. Biochem., 19 (1971) 502–507.

Roisen, F.J., H. Bartfeld, H. Donnenfeld and J. Baxter, Neuron specific in vitro cytotoxicity of sera from patients with amyotrophic lateral sclerosis, Muscle Nerv., 5 (1982) 48–53.

Rowland, L.P. (Ed.), Human Motor Neurone Diseases, Raven Press, New York, 1982.

Shou, L., S.A. Schwartz and R.A. Good, Suppressor cell activity after concanavalin A treatment of lymphocytes from normal donors, J. Exp. Med., 143 (1976) 1100–1110.

Urbaniak, S.J., A.G. White, G.R. Barclay, S.M. Wood and A.B. Kay, Tests of immune function. In: D.M. Weir (Ed.), Handbook of Experimental Immunology, Blackwell Scientific Publications, Oxford, 1978, pp. 47.1–47.31.

Waller, C.A. and I.C.M. MacLennan, Analysis of lymphocytes in blood and tissues. In: R.A. Thompson (Ed.), Techniques in Clinical Immunology, Blackwell Scientific Publications, Oxford, 1977, pp. 170–195.

Westall, R.C., R. Rubin, J. Nieder and C. Jablecki, Low percentage of T cells in amyotrophic lateral sclerosis, Immunol. Lett., 7 (1983) 139–140.

Wolfgram, F. and L. Myers, Effect of serum on anterior horn cells in tissue culture, Science, 179 (1973) 579–580.